Interplay between Mismatch Repair and other Pathways of DNA Metabolism

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Nina

SUMMARY

Mismatch repair (MMR) evolved to repair mismatches that escaped the proofreading activities of replicative DNA polymerases. MMR plays a major role in maintaining genomic stability by preventing accumulation of mutations, and contributes to other DNA repair pathways such as homologous recombination. It also participates in the response to DNA damaging agents.

The subject of my PhD work was to study the role of MMR as an *in vivo* coordinator of cellular responses to genotoxic treatments, in particular to the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), a monofunctional alkylating agent that methylates many macromolecules inside living cells. At clinically–relevant concentrations, the cytotoxicity of MNNG is exerted through the formation of methyl monoadducts on DNA bases. Similar to other S_N1-type alkylating agents, MNNG modifies predominantly the N7 and N3 positions of purine rings. Although N7-methylguanine and N3-methyladenine are the most abundant adducts, they are efficiently removed by base excision repair and, at the concentrations used, do not cause significant cytotoxicity. The single adduct responsible for cell killing is O6-methylguanine (^{6me}G), which comprises only ~8% of all methylations in the DNA. Interestingly, the presence of ^{6me}G in the DNA is not toxic *per se*. Under normal conditions, the methyl group at the O6 position of guanine is rapidly removed by methylguanine methyl transferase (MGMT), which reverts ^{6me}G to G. However, in the absence of MGMT, such as in many tumours, ^{6me}G-containing DNA persists until replication. When the polymerase encounters a ^{6me}G in the template, it preferentially incorporates a thymidine into the nascent strand, thus creating a mismatch. It is believed that MMR-mediated processing of such ^{6me}G/T mismatches ultimately kills the cell, by an unknown mechanism.

In an attempt to elucidate the mechanism of MMR-mediated cell killing, I used the 293T Lα cell line (Cejka et al., 2003), the MMR status of which can be modulated by doxycyclin. In the presence of this drug the cells shut off the expression of hMLH1 and become MMR-deficient and resistant to MNNG treatment. Unexpectedly, low levels of MLH1 expression restored MMR proficiency as assessed by *in vitro* repair assays, but not MNNG sensitivity. In order to sensitize cells to MNNG, the full complement of MLH1 was required. After treatment, such cells arrested in the G_2/M phase of the cell cycle, although the initial levels of DNA damage in both MMR-proficient and -deficient cells was identical.

In order to study the role of MMR proteins in the activation of the cell cycle checkpoint, the 293T Lα cells were treated with MNNG and the subsequent activation of DNA damage signalling pathways was assessed. We showed (Stojic *et al.*) that MNNG treatment induced a cell cycle arrest that was absolutely dependent on functional MMR. Unusually, the MMR-proficient cells arrested only in the second G_2 phase after treatment. Downstream targets of both ATM and ATR kinases were modified, but only the ablation of ATR, or the inhibition of CHK1, attenuated the arrest. The checkpoint activation was accompanied by the formation of nuclear foci containing the signaling and repair proteins ATR, the S*/T*Q substrate, γ-H2AX, and replication protein A (RPA). The persistence of these foci implied that they might represent sites of irreparable damage.

Analysis of the MMR-dependent signaling pathways activated by MNNG suggested that the cell cycle checkpoint was not activated directly. The delay in its activation and the appearance of RPA foci suggested that the initial damage was being processed into secondary lesions that triggered the checkpoint and caused cytotoxicity. I wanted to identify the pathways downstream of the MMR-dependent processing of MNNG damage and learn how they contribute to cell death. I was able to show that the initial recognition of ^{6me}G-containing mispairs by MMR happened in the first S-phase after treatment and this provoked cytologically-visible uncoupling of replication and repair foci. Although ^{6me}G is recognized already in the first cell cycle, its presence is required in both cell cycles after MNNG treatment for cell cycle checkpoint activation, indicating the involvement of another pathway. Activation of the MRN complex was MMR-dependent, but not necessary for the arrest or overall survival. *In vivo* activation of homologous recombination (HR) depended on MMR status and the sites of ongoing recombination contained the ssDNA-binding protein RPA. Lack of HR rendered cells extremely sensitive to MNNG. Moreover, the cell cycle arrest in HR-deficient cells was activated already in the first cycle after treatment. This finding suggests that progression through the first cell cycle requires homologous recombination, possibly to rescue the intermediates created by MMR processing of ^{6me}G-containing mismatches that arose during the first replication.

MMR is highly conserved from bacteria to humans and the sensitivity to ^{6me}G –inducing agents is its hallmark. The finding that MMR status did not affect the sensitivity of *Saccharomyces cerevisiae* to MNNG was therefore unexpected. One possible explanation for this difference was the differential ability of yeast cells to process the secondary lesions

created by MMR. As I showed for mammalian cells, homologous recombination contributes to cell survival after MNNG treatment. Given that HR is much more efficient in yeast as compared to mammalian cells, this difference could account for MNNG resistance in yeast. Indeed, HR inactivation sensitized *S. cerevisiae* to MNNG and, as in human cells, defects in MMR rescued this sensitivity. This finding pays witness to the importance of secondary pathways involved in MMR-mediated cytotoxicity.

ZUSAMMENFASSUNG

Die Fehlpaarungs-Reparatur (MMR) erfüllt die Aufgabe, falsch gepaarte Basen, welche nicht durch die Korrektur-Lesefunktion der replikativen DNA-Polymerasen erkannt werden, zu reparieren. Dieses Reparatur-System spielt eine wichtige Rolle in der Aufrechterhaltung der genomischen Stabilität, indem es Ansammlungen von Mutationen verhindert und massgeblich an anderen DNA-Reparaturwegen wie homologe Rekombination beteiligt ist. Zudem kommt MMR eine grosse Bedeutung in der Antwort auf DNA-schädigende Wirkstoffe zu.

Das Thema meiner Doktorarbeit war es, die Rolle der MMR als *in vivo*-Koordinator der zellulären Antworten auf die genotoxische Behandlung mit dem methylierenden Wirkstoff N-methyl-N'-nitro-N-nitrosoguanidin (MNNG) zu studieren. MNNG ist eine monofunktionale alkylierende Substanz, welche zahlreiche Makromoleküle in der lebenden Zelle methyliert. Die Zell-Toxizität bei der Behandlung mit klinisch relevanten MNNG-Konzentrationen wird durch die Bildung von Methyl-Monoaddukten an DNA-Basen vermittelt. Analog zu anderen SN1-alkylierenden Wirkstoffen modifiziert auch MNNG hauptsächlich die N7- und N3-Positionen von Purinringen. N7-Methylguanin und N3-Methyladenin sind zwar die häufigsten Addukte, werden aber effizient von der Basen-Exzisions-Reparatur repariert und verursachen deshalb keine signifikante Zell-Toxizität. O6-Methylguanin (^{6me}G) hingegen, welches nur für ~8% aller Methylierungen in der Zelle verantwortlich ist, besitzt als einziges Addukt die Fähigkeit, die Zelle zu töten. Interessanterweise ist die Präsenz von ^{6me}G *per se* nicht toxisch. Unter normalen Bedingungen entfernt Methylguanin-Methyltransferase (MGMT) die Methyl-Gruppe an der O6-Position von Guanin rasch und effizient, was eine Revertierung von ^{6me}G zu G zur Folge hat. Fehlt MGMT hingegen, was in manchen Tumoren zu beobachten ist, persistiert die ^{6me}G-enthaltende DNA bis zur Replikation. Wenn die Polymerase auf ein ^{6me}G in der Matrize stösst, wird vorzugsweise ein Thymidin in den wachsenden Strang eingebaut und somit eine Fehlpaarung verursacht. Es wird angenommen, dass MMR solche ^{6me}G/T-Fehlpaarungen prozessiert und die Zelle durch einen bislang unbekannten Mechanismus in letzter Konsequenz zu töten vermag.

Für den Versuch, dem Mechanismus der MMR-abhängigen Zelltötung auf die Spur zu kommen, arbeitete ich mit der 293TLα-Zell-Linie (Cejka et al., 2003), in welcher der MMR-Status mit Hilfe von Doxyzyklin moduliert werden kann. In Anwesenheit dieser Droge wird die Expression von hMLH1 ausgeschaltet, die Zellen werden MMR-defizient

und gleichzeitig resistent gegenüber MNNG. In *in vitro* Reparatur-Versuchen konnte gezeigt werden, dass ein niedriger MLH1-Pegel zwar ausreicht, um die MMR-Kapazität wieder herzustellen, nicht aber um die Empfindlichkeit gegenüber MNNG auszubilden. Um die Zellen wieder MNNG-sensitiv zu machen benötigen diese das ganze Komplement an MLH1.

MMR-profiziente Zellen mit dem vollen Komplement an MLH1, nicht aber MMR-defiziente Zellen, arretieren nach MNNG-Behandlung in der G2/M-Phase des Zellzyklus, obgleich der induzierte DNA-Schaden in MMR-profizienten und MMR-defizienten Zellen identisch ist.

Um die Rolle der MMR-Proteine in der Aktivierung des Zellzyklus-Kontrollpunktes zu studieren wurden 293TLα-Zellen mit MNNG behandelt und die dadurch induzierte Aktivierung des DNA-Schaden-Signalweges beurteilt. Wir konnten zeigen (Stojic et al.), dass die Behandlung mit MNNG eine Zellzyklus-Arretierung auslöst, welche aber ein voll funktionstüchtiges MMR-System voraussetzt. Interessanterweise arretieren MMR-profiziente Zellen nur in der zweiten G2-Phase nach der Behandlung. Nachgeordnete Ziele der beiden Kinasen ATM und ATR sind dabei modifiziert, wobei nur die Ablation von ATR oder die Inhibition von CHK1 die Arretierung abschwächt. Nebst der Kontrollpunkt-Aktivierung konnte die Formation von nuklearen Fokussen beobachtet werden, welche die Signal- und Reparaturproteine ATR, S*/T*Q-Substrat, γ-H2AX und das Replikationsprotein A (RPA) enthalten. Die Persistenz dieser Fokusse deutet an, dass es sich hierbei um Orte mit irreparablem Schaden handeln könnte.

Die Analyse des durch MNNG induzierten MMR-abhängigen Signalweges lässt die Vermutung aufkommen, dass der Zellzyklus-Kontrollpunkt nicht direkt aktiviert wurde. Der Verzug in der Aktivierung und das Auftreten der RPA-Fokusse deutet an, dass der anfängliche Schaden prozessiert und in sekundäre Läsionen umgewandelt wurde, welche ihrerseits den Kontrollpunkt auslösten und die Zell-Toxizität verursachten. Ich wollte die nachgeordneten Wege der MMR-abhängigen Prozessierung von MNNG-induzierten Schäden identifizieren und schlussendlich verstehen, wie dieses Signalnetzwerk den Zelltod beeinflusst. Ich konnte zeigen, dass die Ersterkennung von ^{6me}G-enthaltenden Fehlpaarungen durch die MMR-Maschinerie in der ersten S-Phase nach der Behandlung mit MNNG stattfindet und eine cytologisch sichtbare Abkoppelung der Replikations- und Reparaturfokusse provoziert. Obschon ^{6me}G bereits im ersten Zellzyklus erkannt wird, muss es während beider Zellzyklen nach MNNG-Behandlung vorhanden sein, um den Zellzyklus-Kontrollpunkt aktivieren zu können, was für eine zusätzlich involvierte

Signalkaskade spricht. Aktivierung des MRN-Komplexes ist MMR-abhängig, aber weder für die Arretierung noch das gesamte Überleben notwendig. *In vivo*-Aktivierung von homologer Rekombination (HR) hängt vom MMR-Status ab und Orte mit aktiver Rekombination enthalten das an ssDNA bindende Protein RPA. Zellen ohne funktionstüchtige HR sind extrem empfindlich gegenüber MNNG. Zudem ist die Zellzyklus-Arretierung in HR-defizienten Zellen bereits im ersten Zellzyklus nach der Behandlung aktiviert. Diese Beobachtung lässt vermuten, dass homologe Rekombination für die Progression durch den ersten Zellzyklus nötig ist, möglicherweise um Zwischenprodukte, welche durch die MMR-Prozessierung von ^{6me}G-enthaltenden Fehlpaarungen während der ersten Replikation entstehen, zu retten.

MMR ist hochkonserviert in Bakterien bis zum Menschen und trägt das Kennzeichen hoher Empfindlichkeit gegenüber ^{6me}G-induzierenden Wirkstoffen. Deshalb erstaunt die Tatsache, dass in *Saccharomyces cerevisiae* der MMR-Status keinerlei Einfluss auf die MNNG-Sensitivität hat. Eine mögliche Erklärung für diese Diskrepanz ist die unterschiedliche Fähigkeit von Hefezellen, sekundäre MMR-Läsionen zu prozessieren. Konsistent mit meinen Resultaten in Säugerzellen trägt homologe Rekombination massgeblich zum Überleben der Zelle nach Behandlung mit MNNG bei. Da HR in Hefezellen verglichen mit Säugerzellen sehr viel effizienter ist, könnte dieser Unterschied die MNNG-Resistenz in Hefe erklären. In der Tat reagiert *S. cerevisiae* mit inaktivierter HR sehr empfindlich auf MNNG und – analog zu den menschlichen Zellen – kann diese Sensitivität mit einem Defekt im MMR-System gerettet werden. Dieses Resultat zeigt deutlich die Wichtigkeit der sekundären Signalwege, welche in die MMR-abhängige Zell-Toxizität involviert sind.

1 INTRODUCTION

"The possibility that genes were subject to the hurly-burly of both insult and clumsy efforts to reverse the insults was unthinkable."

Frank Stahl
(Friedberg, 1997)

Since the perception of DNA as a highly stable macromolecule changed, major insights into the metabolism of the "molecule of life" have been discovered. Indeed, it became apparent that DNA in all living organisms is constantly subjected to alterations by endogenous metabolites and exogenous DNA-damaging agents. More interestingly, studies of three "R's" of DNA metabolism – Replication, Recombination and Repair revealed the existence of ingenious mechanisms for tolerating and repairing the damage, thus making DNA the only biomolecule that is specifically *repaired* – all others being *replaced*.

The importance of repair mechanisms becomes evident in multiple human syndromes related to inefficient repair of certain types of DNA damage. Failure of these mechanisms can lead to serious consequences (Table 1) but also provide valuable insights into the detailed mechanisms of their actions.

DNA damage types include covalent changes of DNA structure and non-covalent anomalous structures, including base-pair mismatches, loops and bubbles arising from strings of mismatches. Some of these can arise during normal cell growth and replication, while others are usually consequences of the action of exogenous agents. During replication, recombination and repair, fork structures, bubbles, Holliday structures, stalled and collapsed replication forks and other non-duplex DNA forms are generated, providing high-affinity sites for repair proteins.

Oxidation, alkylation, deamination, depurination and depyrimidation of DNA create ~10^4 mutagenic DNA base lesions per day in each human cell (Lindahl, 1993). Damage to DNA bases occurs constantly and it can stem from numerous sources: by-products of natural aerobic respiration, environmental chemicals or chemotherapeutic drugs, ultraviolet (UV) light or ionizing radiation (IR). Besides creating reactive oxygen species, UV radiation gives raise to specific products such as cyclobutane pyrimidine dimmers and (6-4) photoproducts. Chemicals form various base adducts, either bulky ones, such as those generated by reaction with large polycyclic hydrocarbons or simple ones, such as

6-methylguanine, 3-methyladenine or 7-methylguanine generated by alkylating agents. Large proportion of chemotherapeutic drugs, including cisplatin, mitomycin C, psoralen, nitrogen mustard and adriamycin, form base adducts.

DNA backbone damage includes abasic sites and single and double strand breaks. Abasic sites are generated spontaneously, by the formation of unstable base adducts or by base excision repair. Single-strand breaks are produced either directly by damaging agents, or single-strand breaks and gaps in the range of 1-30 nucleotide are produced as intermediates of base- and nucleotide excision repair. Ionizing radiation and other DNA-damaging agents form double-strand breaks. In addition, double-strand breaks are essential intermediates in recombination and perhaps during transposition.

Bifunctional agents such as cisplatin, nitrogen mustards, mitomycin C, and psoralen form interstrand cross-links and DNA-protein cross-links.

As the molecule that guards the information necessary for the life of the cell, DNA undergoes many repair processes, which ensure its integrity. On the other hand, evolution requires genetic diversification, so mutations, another important consequence of DNA damage, must be promoted at levels that will allow evolutionary changes. DNA thus balances between a requirement for genomic stability and a necessity for instability.

In the next paragraphs I will discuss major DNA repair pathways with more detailed description of mismatch repair and homologous recombination as the major pathways studied during my PhD project.

Table 1. Human syndromes with defective genome maintenance mechanisms. (Hoeijmakers, 2001)

SYNDROME	***Affected pathway / gene***	***Main type of genome instability***	***Major Cancer predisposition***
Xeroderma pigmentosum (XP)	NER, TCR / XPA-XPG	Point mutations	UV-induced skin cancer
Cockayne syndrome (CS)	TCR / CSA, CSB	Point mutations	None
Trichothiodystrophy (TTD)	NER / TCR	Point mutations	None
Ataxia telangiectasia (AT)	DSB repair / ATM	Chromosome aberrations	Lymphomas
AT-like disorder (ATLD)	DSB repair / MRE11	Chromosome aberrations	Lymphomas
Nijmegen breakage syndrome (NBS)	DSB repair / NBS1	Chromosome aberrations	Lymphomas
Familial breast cancer predisposition	Recombination, DSB repair / BRCA1/2	Chromosome aberrations	Breast and ovarian cancer
Werner syndrome (WS)	DSB repair / WRN	Chromosome aberrations	Various cancers
Bloom syndrome (BS)	Recombination, DSB repair / BLM	Chromosome aberrations / SCE	Leukaemia, Lymphomas
Rothmund-Thompson syndrome (RTS)	Recombination, DSB repair / RECQL4	Chromosome aberrations	Osteosarcoma
Ligase IV deficiency	NHEJ / DNA ligase IV	Recombination fidelity	Leukaemia
Fanconi anaemia (FA)	DNA cross-link repair / FANCA-G	Chromosome aberrations	Leukaemia
Seckel syndrome	DNA damage checkpoint / ATR		None
Hereditary non-polyposis colon cancer (HNPCC)	MMR / MSH2/6 and MLH1/PMS2	Microsatelite instability (MI)	Colorectal cancer
Xeroderma pigmentosum variant (XPV)	TLS / DNA polymerase η		UV-induced skin cancer
Radiosensitive severe combined immunodeficiency	NHEJ / Artemis	Chromosome aberrations	Lymphomas

1.1 DNA DAMAGE REPAIR PATHWAYS

In order to protect their genomes from the deleterious consequences of DNA damage, cells have evolved multiple pathways that help coordinate cell growth, division and removal of the damage. General effect and consequence of DNA damage can be categorized into four types of responses (Figure 1): DNA repair, DNA damage checkpoints, transcriptional response and apoptosis (Sancar et al., 2004). Defects in any of these pathways may cause genomic instability. Each of these four pathways should not be taken as independent order of events, but rather as interconnected networks.

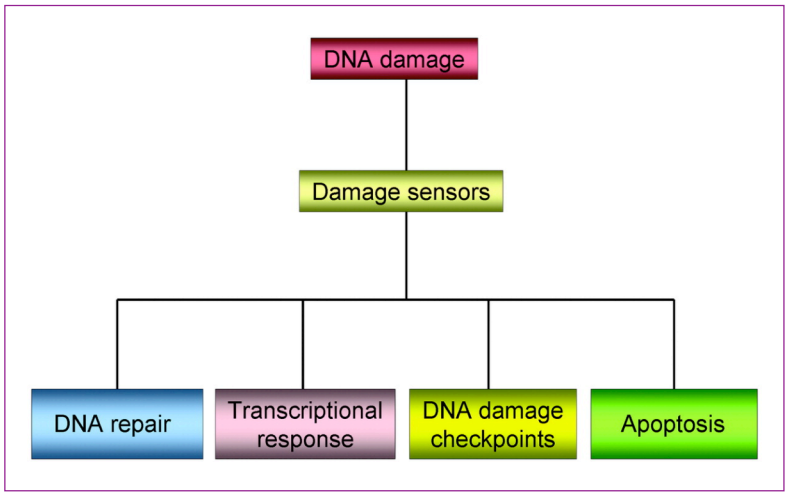


Figure 1. DNA damage response reactions in mammalian cells.
The four responses (DNA repair, transcriptional response, DNA damage checkpoints, and apoptosis) may function independently, but frequently a protein primarily involved in one response may participate in other responses.

In contrast to enzymes with simple substrates, proteins that bind to a specific sequence or structure in DNA, must recognize their targets in a vast excess of related structures. Damage sensors not only bind undamaged DNA in search of damage, but they also contact undamaged DNA during specific binding. Therefore, they generally have non-negligible affinity for undamaged DNA. Since the amount of undamaged DNA vastly exceeds that of damaged DNA, DNA damage sensors spend far more time associated with undamaged DNA than with damaged DNA. Yet, these sensors carry out their specific functions in the presence of high concentrations of non-specific DNA, because damage recognition is usually a multistep reaction. There is thus a low probability that all of the steps will occur subsequent to the initial contact with undamaged DNA. However, even with multistep recognition, the discrimination between undamaged and damaged

DNA is not absolute, so that neither DNA repair nor the DNA damage checkpoints should be envisioned as operated by molecular switches. Rather, both processes are operative at all times, but the magnitudes of the repair or checkpoint reactions are amplified by the presence of DNA damage (Sancar et al., 2004).

Depending on the type of DNA damage, different repair pathways are activated. Examples of abnormal DNA structures that induce such responses are shown in Figure 2.

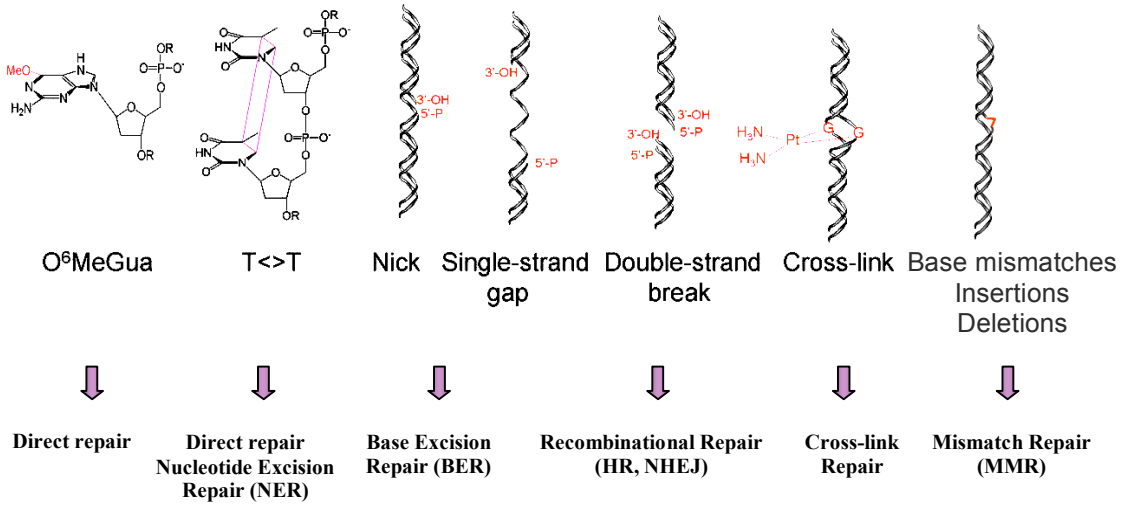


Figure 2. DNA lesions and structures that elicit DNA response reactions

Repair mechanisms can be grouped into five categories: direct repair of damage, Base Excision Repair (BER), Nucleotide Excision repair (NER), Mismatch Repair (MMR), double-strand break repair and although not repair *per se*, DNA damage bypass.

In some cases, certain types of adducts can be addressed by more than one repair pathway and sometimes two different pathways might be necessary to fully repair the damage. Repair reactions *in vivo* are usually multistep processes with highly interconnected pathways.

1.1.1 Direct repair

Direct repair, or direct reversal is the simplest mechanism, which involves a single enzyme reaction for removal of certain types of DNA damage. Although it might seem that reversal of damage would be the easiest way to correct the damage, in most cases the reverse reaction is not possible for thermodynamic or kinetic reasons. In addition, direct reversal mechanisms of DNA repair require that each type of chemical alteration be addressed by a dedicated enzyme. What the cell needs are more general mechanisms capable of correcting different sorts of chemical damage with a limited toolbox. This requirement is met by the mechanisms of excision repair.

Maybe that's the reason why there are only four examples of the direct repair enzymes: photoreactivation by photolyases, alkyl transfer by DNA methyl transferases, oxidative demethylation and ligation. Photolyase is not present in many species, including humans, whereas methylguanine DNA methyltransferase has nearly universal distribution.

The third example of direct damage reversal is provided by AlkB, a protein that is found in most living organisms, including humans. This protein is a member of the class of enzymes called alpha-keto-glutarate-dependent and iron-dependent oxygenases (aKG-Fe(II)-oxygenases), which use iron-oxo intermediates to oxidize chemically inert compounds. In the process, alpha-keto-glutarate is converted to succinate and CO_2. AlkB is capable of reversing methylation at the 1 position of adenine and the structurally similar 3 position of cytosine. The reaction catalyzed by AlkB couples the oxidative decarboxylation of alpha-keto-glutaric acid to the hydroxylation of the methyl group. The methyl group then spontaneously decomposes to formaldehyde, restoring the original base (Begley and Samson, 2003).

A final example of direct damage reversal is the sealing of a subset of nicks in DNA by DNA ligases. Of course, DNA ligases can only seal nicks having 5'-phosphates and 3'-hydroxyls. Nicks with other configurations, or nicks accompanied by additional backbone or base damage, require more complicated processing prior to repair and would not be classified as direct damage reversal mechanisms.

Photoreactivation

Many species from each of the three kingdoms of life and even some viruses have photolyases, whereas other species do not (Sancar, 2003). There are two types of photolyases, one that repairs cyclobutane pyrimidine dymers (photolyase) and the other that repairs (6-4) photoproducts (6-4 photolyase). CPD photolyases are found in bacteria, fungi, plants and many vertebrates, but not in placental mammals. In addition, 6-4 photolyases (which repair 6-4PPs) have been found in insects, reptiles and amphibians, but not in *E. coli*, yeast or mammals.

The reaction catalyzed by CPD photolyases was the first DNA repair process to be discovered (Dulbecco, 1949; Kelner, 1949), well before the double-helical structure of DNA was described in 1953.

CPD photolyase contains two chromophores responsible for absorbing light energy. In all such photolyases, one of the chromophores is FADH- and the other is either methenyl-tetrahydrofolate (MTHF) or 8-hydroxy-5-deazaflavin (8-HDF) (Thompson and Sancar, 2002). MTHF and 8-HDF act as primary light gatherers, transferring energy to FADH-. The energy from FADH- is then used to split the dimer (Figure 3). Placental mammals lack photolyases but contain two proteins with high sequence and structural similarities to photolyases, but no repair function. These proteins, named cryptochromes, function as photoreceptors for setting the circadian clock (Sancar, 2003).

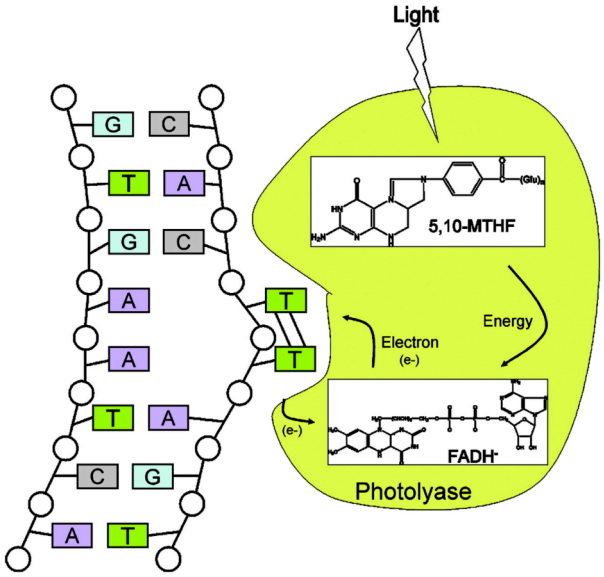


Figure 3. Photoreactivation reverses DNA damage. DNA exposed to ultraviolet (UV) radiation results in covalent dimerization of adjacent pyrimidines, typically thymine residues (thymine dimers). These lesions are recognized by a photoreactivating enzyme, which absorbs light at wavelengths >300nm (such as fluorescent light or sunlight) and facilitates a series of photochemical reactions that monomerize the dimerized pyrimidines, restoring them to their native conformation

Alkyl transfer

Another example of direct damage reversal is repair of O6-alkyl guanine by transfer of the methyl group from the DNA to a cysteine in a protein, an O6-alkylguanine-DNA alkyltransferase. This is a small protein of ~20 kDa that does not contain a cofactor and is ubiquitous in nature. Like photolyases, it is presumed to recognize damage by three-dimensional diffusion and, after forming a low-stability complex with the DNA backbone at the damage site, it is thought to flip-out the 6-methylguanine base into the active site cavity (Daniels and Tainer, 2000), wherein the methyl group is transferred to an active site cysteine (Lindahl et al., 1988). The protein then dissociates from the repaired DNA, but the C-S bond of methylcysteine is stable and therefore, after one catalytic event, the enzyme becomes inactivated and is accordingly referred to as a suicide enzyme (Figure 4).

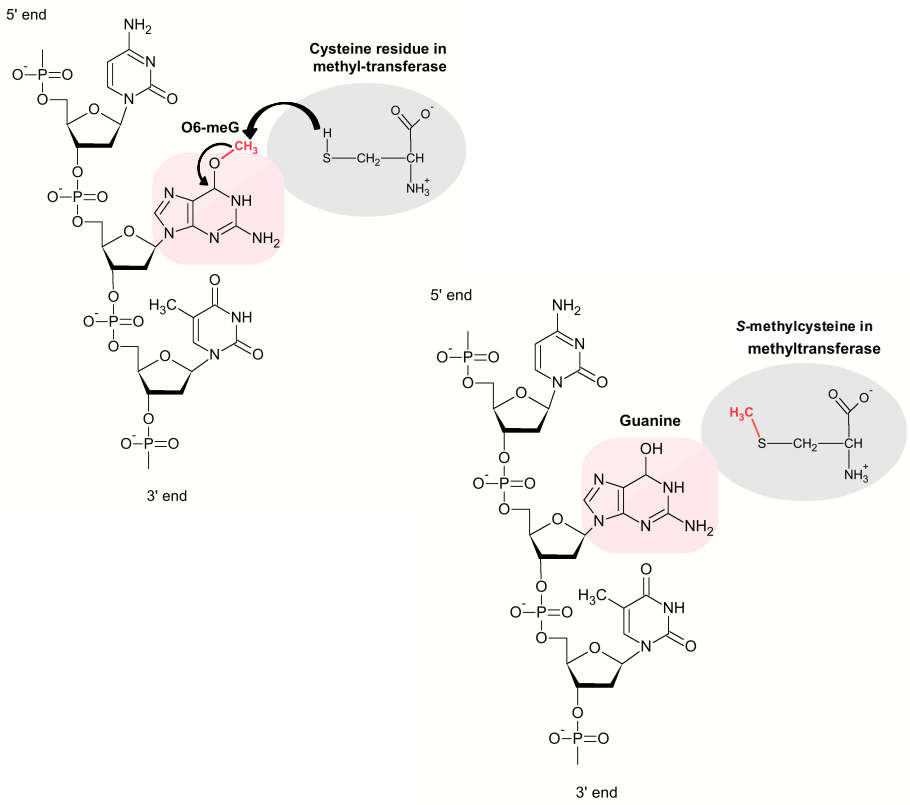


Figure 4. MGMT mechanism.
Transfer of an alkyl group from an O-6 position of guanine to an internal cysteine permanently inactivates the enzyme and targets it for proteosomal degradation.

1.1.2 Base excision repair

Base excision repair (BER) works mainly on DNA damage that arises spontaneously in a cell as a result of hydrolytic events such as deamination or bases loss, fragmentation of bases by ionizing radiation and oxidative damage or methylation of ring nitrogens by endogenous agents. The BER pathway is the most important cellular protection mechanism responding to oxidative DNA damage, generated by reactive oxygen species formed during normal metabolism or by exogenous agents (Tuteja and Tuteja, 2001). Specificity of the repair is determined by the glycosylase employed in the initial step of recognition and the hydrolysis of the N-glycosyl bond linking the modified or damaged base to the deoxyribose-phosphate chain (Table 2). Nevertheless, all of the variant pathways have features in common and each of the pathways can be considered to consist of 3 steps:

- Removal of the aberrant base by an appropriate DNA N-glycosylase to create an AP site
- Nicking of the damaged DNA strand by AP endonuclease upstream of the AP site, thus creating a 3'-OH terminus adjacent to the AP site
- Extension of the 3'-OH terminus by a DNA polymerase, accompanied by excision of the deoxyribose-phosphate (dRp)

Table 2. Some of the human DNA glycosylases (Christmann et al., 2003; Schoerer and Jiricny, 2001)

Acronym	*Full Name*	*AP Lyase Activity*	*Substrates*
UNG	Uracil DNA N-Glycosylase	No	ssU>U:G>U:A, 5-FU
TDG	Thymine DNA Glycosylase	No	U:G>ethenocytosine:G>T:G
UDG2	Uracil DNA Glycoslyase 2	No	U:A
SMUG1	Single-strand-selective Monofunctional Uracil-DNA Glycosylase 1	No	ssU>U:A, U:G
MBD4	Methyl-CpG-binding Domain 4	No	U or T in U/TpG:5-meCpG
MPG	Methyl Purine DNA Glycosylase	No	3-meA, 7-meA, 3-meG, 7-meG
MYH	MutY Homolog	No	A:G, A:8-oxoG
OGG1	8-Oxo-Guanine Glycosylase 1	Yes	8-oxoG:C
NTH1	Endonuclease Three Homolog 1	Yes	T-glycol, C-glycol, formamidopyrimidine

In addition to removing altered bases, some DNA glycosylases also possess an AP lyase activity, which allows them to cleave the DNA backbone on the 3' side of the AP site (Figure 5). The cleavage is between C and O, not between O and P, thus, regardless of whether the DNA glycosylase possesses a lyase activity, the next step in BER is catalyzed by an AP endonuclease, which cleaves the DNA backbone on the 5' side of the AP site. The insertion of the first nucleotide is not dependent on the chemical structure of the AP site. During short-patch BER, the 5'-dRP is displaced by DNA polymerase β (Polβ), which inserts a single nucleotide. The critical step in the decision between short- and long-patch BER is the removal of the 5'-dRP upon the insertion of the first nucleotide. Besides polymerisation activity, Polβ also possesses a lyase activity and is thereby able to catalyze the release of the hemiacetal form of 5'-dRP residues from incised AP sites by β-elimination. In contrast, oxidised or reduced AP sites, 3'-unsaturated aldehydes or 3'-phosphates are resistant to β-elimination by Polβ. Upon dissociation of Polβ from the DNA, the repair process is completed by DNA-Ligase, which seals the remaining nick. During long-patch BER, strand displacement and further DNA synthesis is accomplished after dissociation of Polβ by Polε or Polδ together with PCNA and RF-C, resulting in repair patches of up to 10 nucleotides. The removal of the deoxyribosephosphate flap structure (5'-dRPflap) is executed by the flap endonuclease FEN1, which is stimulated by PCNA. Ligase I interacts with PCNA and Polβ and participates mainly in long-patch BER. DNA ligase III interacts with XRCC1, Polβ and PARP-1 (poly(ADP-ribose) polymerase-1) and is involved only in short-patch BER (Christmann et al., 2003; Huffman et al., 2005)

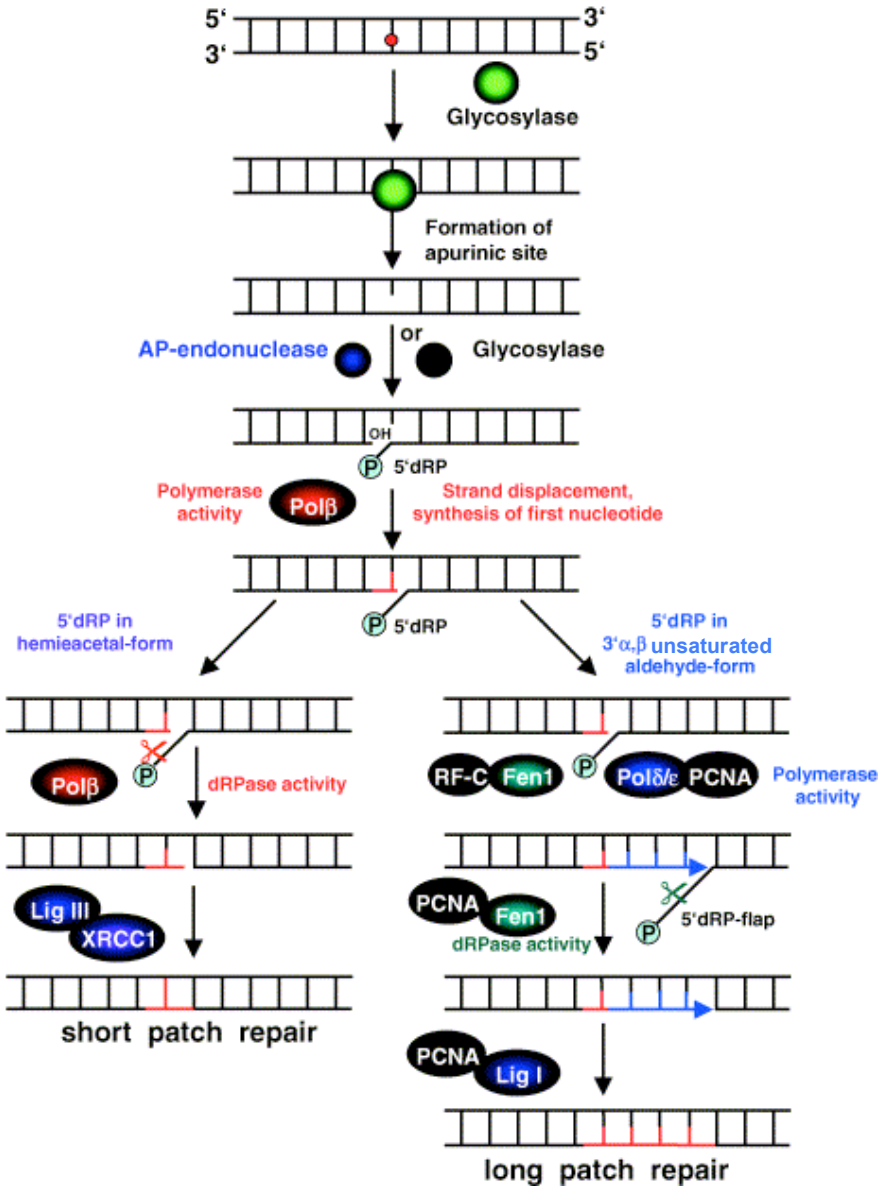


Figure 5. Mechanism of base excision repair (BER).
Recognition of the DNA lesion occurs by a specific DNA glycosylase, which removes the damaged base by hydrolyzing the N-glycosidic bond. The remaining AP site is processed by APE. Depending on the cleavability of the resulting 5'-dRP by Polβ, repair is accomplished by the short or long patch BER pathway. Modified from Christmann et al., 2003.

1.1.3 Nucleotide Excision Repair

Nucleotide excision repair (NER) is the major repair system for removing bulky DNA lesions formed by exposure to radiation or chemicals, or by protein addition to DNA. The main function of the nucleotide excision repair pathway is the removal of UV-induced lesions. The damaged bases are removed by the "excision nuclease," a multisubunit enzyme system that makes dual incisions bracketing the lesion in the damaged strand (Figure 6). Because of the wide substrate range, excision repair cannot recognize the specific chemical groups that make up the lesion, but is thought to recognize the phosphodiester backbone distortion created by the damage. The basic steps of nucleotide excision repair are (a) damage recognition, (b) dual incisions bracketing the lesion to form a 12-13-nt oligomer in prokaryotes or a 24-32-nt oligomer in eukaryotes, (c) release of the excised oligomer, (d) repair synthesis to fill in the resulting gap, and (e) ligation. In humans, excision repair is carried out by 6 repair factors (RPA, XPA, XPC, TFIIH, XPG, and XPF/ERCC1) and defects in excision repair cause a photosensitivity syndrome called Xeroderma pigmentosum (XP), which is characterized by a very high incidence of light-induced skin cancer.

NER can be subdivided into two distinct pathways termed global genomic repair (GGR) and transcription-coupled repair (TCR) (Figure 6). GGR is thought to be largely transcription-independent and removes lesions from the non-transcribed domains of the genome and the non-transcribed strand of transcribed regions. 6-4PPs, which distort the DNA more than CPDs, are removed rapidly and predominantly by GGR. In contrast, CPDs are removed very slowly by GGR. Their removal occurs more efficiently by TCR from the transcribed strand of expressed genes. Defects in TCR are directly linked to Cockayne's syndrome, which involves two complementation groups: CSA and CSB. CSA and CSB, as well as XPB, XPD (as part of TFIIH) and XPG protein are essential for TCR (Cleaver, 2005). In contrast to TCR, the mechanism of GGR has been elucidated in great detail.

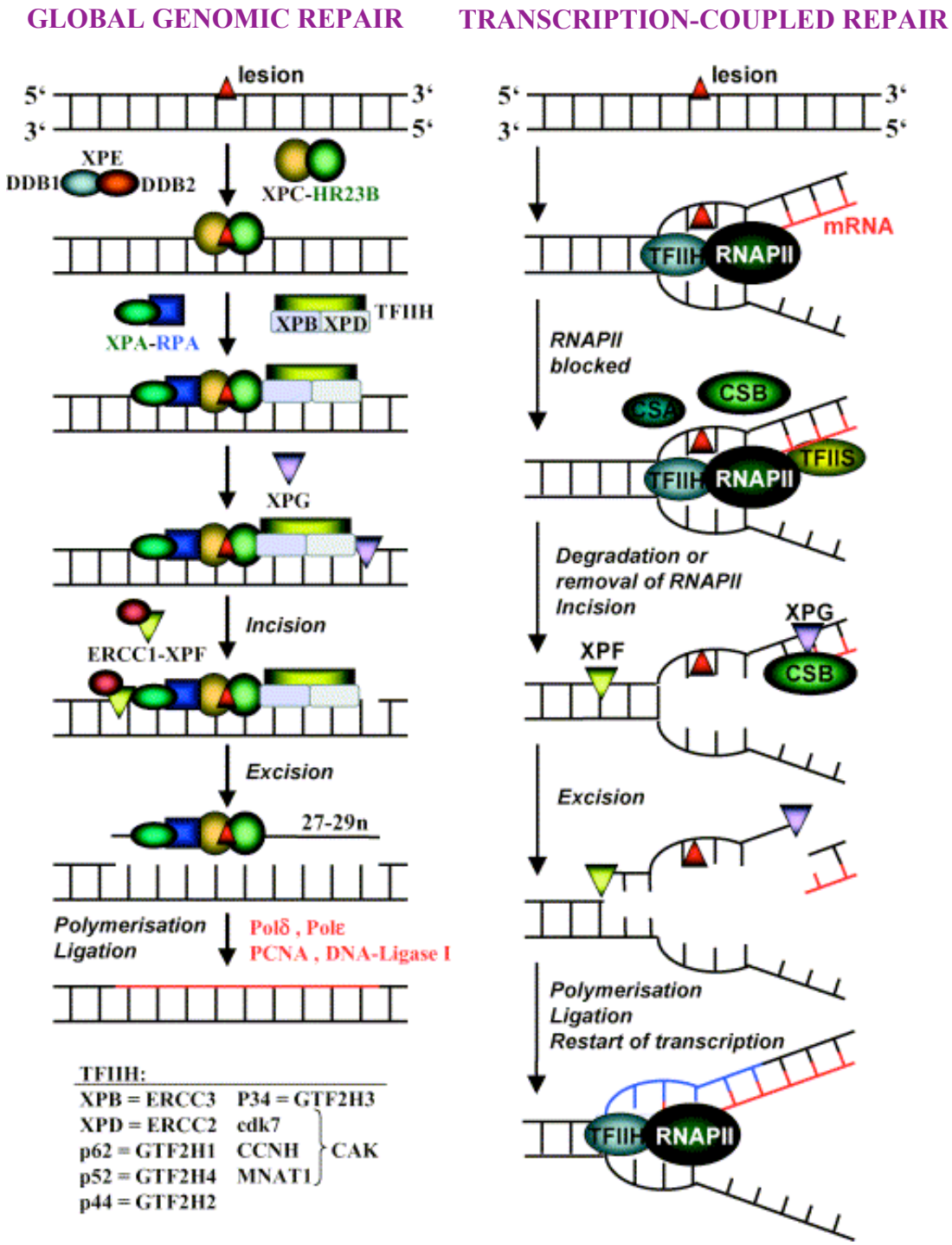


Figure 6. Mechanism of nucleotide excision repair (NER).
During global genomic repair (GGR), recognition of the DNA lesion occurs by XPC–HR23B, RPA–XPA or DDB1–DDB2. DNA unwinding is performed by the transcription factor TFIIH and excision of the lesion-containing nucleotide by XPG and XPF–ERCC1. Finally, resynthesis occurs by Polδ or Polε and ligation by DNA ligase I. During transcription-coupled repair (TCR), the lesion blocks RNA-Polymerase II (RNAPII). This leads to assembly of CSA, CSB and/or TFIIS at the site of the lesion, by which RNAPII is removed from the DNA or displaced from the lesion, making it accessible to the exonucleases XPF–ERCC1 and XPG, cleaving the lesion-containing DNA strand. Resynthesis again occurs by Polδ or Polε and ligation by DNA ligase I.

1.2 MISMATCH REPAIR

In contrast to lesions produced within the DNA helix by chemical damage, mismatched base pairs arise during the course of normal DNA metabolism such as replication or recombination. Mismatch repair (MMR) is responsible for correcting base-base mismatches as well as insertion-deletion loops (IDLs) that might arise during replication due to polymerase slippage, most often on repetitive sequences.

MMR participates in a wide variety of DNA transactions and tits inactivation can have either beneficial or deleterious consequences for the organism. The major consequence of defective MMR is an increased rate of point mutations, resulting from unrepaired errors of replicative polymareses. Loss of MMR pathway in bacteria favours survival and exchange of genetic material under stress conditions (Matic et al., 2003), while in mammals MMR deficiency contributes to initiation and promotion of tumours (Loeb et al., 2003). The error rate of the replicative polymerase δ is ~1 misincorporation and 1 -2 deletions per 10^5 nucleotides (Kunkel, 2003). When addressed by MMR, replication fidelity approaches 1 error per 10^8 to 10^9 nucleotides (Sarasin, 2003). In addition to recognizing replication-generated mismatches, MMR proteins also recognize mismatches in heteroduplex recombination intermediates. These elicit either a repair process that leads to a genetically detectable gene conversion event, or trigger an anti-recombination activity that prevents the recombination event from going to completion. The anti-recombination activity of MMR proteins promotes genome stability by inhibiting interactions between diverged sequences present in a single genome or derived from different organisms. Besides undamaged mismatches, the MMR machinery also recognizes certain DNA lesions generated by normal intracellular metabolism such as oxidative stress (Colussi et al., 2002) or modifications due to chemical treatments. Some chemotherapeutic drugs rely on the activity of MMR proteins for the activation of cell cycle checkpoints and apoptosis (Stojic et al., 2004). The role of MMR proteins in maintaining genomic stability is highlighted by the finding that mutations in MMR genes lead to hereditary non-polyposis colorectal cancer (HNPCC) (Lynch et al., 1993). The proteins involved in MMR are listed in Table 3. Besides proteins specific for MMR only, there are others, which participate in DNA metabolism required for efficient MMR, such as proteins involved in replication and recombination. MMR is greatly conserved mechanism, from bacteria to humans, and outlines of the mechanism can be generalized.

Two systems will be described, methyl-directed repair in *E. coli* and human mismatch repair.

Table 3. MMR proteins
Identities and functions of *Escherichia coli* and eukaryotic proteins involved in MMR of replication errors (Kunkel and Erie, 2005)

E. coli protein	***Function***	***Homologs***	***Function***
MutS	Binds mismatches	**MSH2-MSH6 (MutSα)**	Binds single base-base and 1-2 base IDL mismatches
		MSH2-MSH3 (MutSβ)	Binds of some single base IDLs and IDLs 2 bases Partially redundant with Msh2-Msh6
MutL	Matchmaker that coordinates multiple steps in MMR	**MLH1-PMS2 (yPms1) (MutLα)**	Matchmaker for coordinating events from mismatch binding by MutS homologs to DNA repair synthesis
		MLH1-MLH2 (hPMS1) (MutLβ)	Function of human heterodimer unknown. Suppresses some IDL mutagenesis in yeast
		MLH1-MLH3 (MutLγ)	Suppresses some IDL mutagenesis Participates in meiosis
MutH	Nicks nascent unmethylated strand at hemimethylated GATC sites	**None**	
γ-δ Complex	Loads β-clamp onto DNA	**RFC complex**	Loads PCNA, modulates excision polarity
β-Clamp	Interacts with MutS and may recruit it to mismatches and/or the replication fork Enhances processivity of DNA pol III	**PCNA**	Interacts with MutS and MutL homologs. Recruits MMR proteins to mismatches. Increases MM binding specificity of Msh2-Msh6 Participates in DNA repair synthesis
Helicase II	Loaded onto DNA at nick by MutS and MutL Unwinds DNA to allow excision of ssDNA	**None**	
ExoI ExoX	Perform 3' to 5' excision of ssDNA	**EXOI (Rth1)**	Excision of dsDNA
RecJ ExoVII	Perform 5' to 3' excision of ssDNA (also 3' to 5' excision by ExoVII)	**3' exo of Polδ**	Excision of ssDNA Synergistic mutator with Exo1 mutant
DNA pol III	Accurate resynthesis of DNA	**DNA polδ**	Accurate repair synthesis
SSB	Participates in excision and repair synthesis	**RPA**	Participates in excision and in DNA synthesis
DNA ligase	Seals nicks after completion of repair synthesis	**DNA ligase**	Seals nicks after completion of DNA synthesis

1.2.1 Methyl-directed mismatch repair in *E.coli*

The components of bacterial MMR system were documented in 1980 by analysis of strains hypersensitive to 2-aminopurine (Glickman and Radman, 1980) although the existence of mismatch repair has been known before (Witkin, 1975, Wildenberg, 1975). The *E. coli* MMR system has been completely reconstituted in vitro (Lahue et al., 1989) and involves three mismatch proteins: MutS, MutL, and MutH. MutS is an ATPase that effects mismatch recognition. MutL is an ATPase that couples mismatch recognition by MutS to downstream processing steps, and MutH is a methylation-sensitive endonuclease that targets repair to the newly synthesized DNA strand. Inactivation of bacterial MMR system results in a strong mutator phenotype, which arises from errors in the newly synthesized strand of DNA (Glickman et al., 1978; Herman and Modrich, 1981).

Strand discrimination in the *E. coli* MMR system is made possible by the transient unmethylation of newly synthesized DNA, which lags behind replication some 2-5 minutes. Specifically, the MutH protein cleaves the unmethylated strand of a hemimethylated GATC *dam* methylation site, thereby marking the nicked strand for exonucleolytic degradation and resynthesis. Incision is mismatch dependent, with the MutH endonuclease being activated *in vitro* by a complex of MutS, MutL and mismatched DNA (Au et al., 1992). MutH can nick DNA on either side of a mismatch and, depending on which side the cleavage occurs, either a single-strand specific 5'→3' or a 3'→5' exonuclease degrades the mismatch-containing nicked strand. In the *E. coli* system, there are two 5'→3' exonucleases (exonuclease VII and the RecJ exonuclease) and two 3'→5' exonucleases (exonuclease I and exonuclease X) that can effect the degradation of the nicked strand. Unwinding by the UvrD helicase begins at the nick and proceeds in a directional manner toward and past the mismatch. The methyl-directed nature of the *E. coli* MMR system is an efficient way to discriminate template and daughter strands during DNA replication. MutL has been speculated to load the UvrD helicase onto the nick in a directional manner such that DNA unwinding proceeds toward the mismatch. MutL coordinates the mismatch binding activity of MutS with the MutH cleavage and UvrD helicase activities, and thereby directs the strand removal process (Harfe and Jinks-Robertson, 2000). Resynthesis is mediated by the DNA polymerase III holoenzyme with the help of single-strand binding protein (SSB). The nick is ligated by DNA ligase (Figure 7) (Jiricny, 1998; Li, 2003).

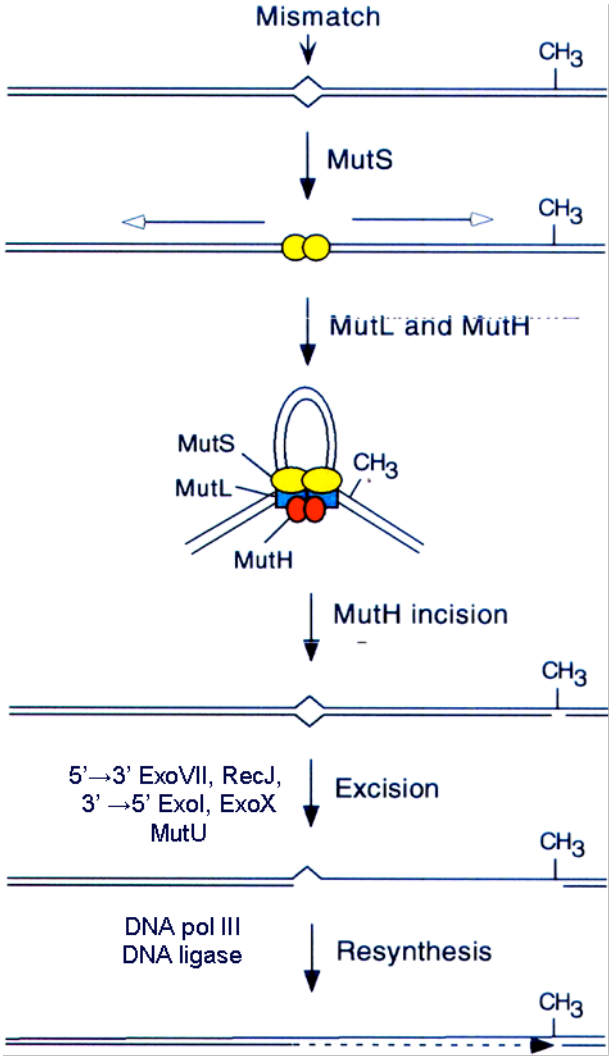


Figure 7. Mechanism of mismatch correction in *E.coli*.
The mispair that arose as an error of a DNA polymerase is present in a DNA heteroduplex, which is transiently unmethylated at GATC sequences in the newly replicated strand. Binding of the MutS protein to the mismatch initiates ATP-dependent conformational change of MutS, followed by a movement of the bound protein away from the mispair, while ATP hydrolysis drives the bi-directional movement of the DNA through the bound MutS, thus forming a loop. At the same time, a multiprotein complex containing MutS and MutL homodimers and two molecules of the strand specific endonuclease MutH, assembles at the base of the looped structure. The assembly of the complex activates the endonucleolytic activity of MutH, which cleaves the newly synthesized DNA 5' at the unmethylated GATC sequence. The cleaved strand is then degraded from the nick up to and slightly past the mismatch site, either by ExoVII or RecJ (in cases where the unmethylated GATC was situated 5' from the mispair), or by ExoI or ExoX (when the nick was 3' from the mispair). The single-stranded region thus generated is protected by the single strand-binding protein Ssb. Polymerase III holoenzyme fills the gap and DNA ligase repairs the nick (Jiricny, 1998). Figure adopted from Harfe and Jinks-Robertson, 2000.

1.2.2 Eukaryotic mismatch repair system

Eukaryotic MMR has features in common with *E. coli* MMR, but the proteins involved in the repair pathway (Table 3) can differ, depending on the nature of the mismatch and the substrate for excision. Repair is initiated when complexes of MutS homologs, either MSH2-MSH6 (MutSα) or MSH2-MSH3 (MutSβ), bind to a mismatch. The choice of the complex binding to the mismatch depends on the structure of the mismatch itself: base-base mismatches and insertion-deletion loops with 1 or 2 nucleotides are recognized primarily by MutSα, while larger insertion-deletion loops, composed of 6-12 nucleotides are preferentially bound by MutSβ (Karran, 1995). The relative amounts of these two complexes *in vivo* are balanced in such a way that the amount of MutSβ is 6-8 times lower than MutSα. Both MSH6 and MSH3 proteins are stabilised by interaction with MSH2. Therefore, deficiency of MSH2 brings about destabilization of both MSH6 and MSH3.

MutSα not only binds to spontaneously-occurring base mismatches, but also to various chemically-induced DNA lesions such as alkylation-induced O^6-methylguanine paired with cytosine or thymine, 1,2-intrastrand (GpG) cross-links generated by cisplatin, purine adducts of benzo pyrene-7,8-dihydrodiol-9,10-epoxides, 2-aminofluorene or *N*-acetyl-2-aminofluorene, and 8-oxoguanine (Christmann et al., 2003). The model of human mismatch repair will be described using a base-base mismatch (MutSα system) as an example, but the same mechanism is thought to occur in the MutSβ system (Figure 9.)

The recognition of mismatches or chemically-modified bases is mediated by the MutSα, comprised of MSH2 (Fishel et al., 1993) and MSH6 (Palombo et al., 1995), which binds to the lesion. It was reported that the binding of MutSα is accompanied and enhanced by phosphorylation (Christmann et al., 2002).

Presently, it is not clear how MMR discriminates between the parental and the newly synthesized DNA strand. One possibility could be through recognition of discontinuities in the daughter strands, either in the form of gaps between Okazaki fragments or the 3' terminus of the leading strand. In order to couple the mismatch and the nicks, which might be some distance apart, the recognition complex of MutSα must translocate along the DNA away from the mismatch. There are two models describing the translocation of MutSα: the first has been proposed by Modrich (Blackwell et al., 1998a; Blackwell et al., 1998b) and according to this hydrolysis-driven translocation, MutSα uses the energy gained by ATP hydrolysis to translocate actively along the DNA from the site of mismatch recognition to a site responsible for signalling the strand specificity (Figure

8A). The second, the so called "molecular switch" model has been proposed by Fishel and colleagues (Gradia et al., 1997; Gradia et al., 1999) and suggests that MutSα binds the mismatch in its ADP form ("active state"). Mismatch triggers an ADP→ATP transition, which brings about a conformational change and the formation of hydrolysis-independent sliding clamp. The hydrolysis of ATP provokes its dissociation from the DNA (Fishel, 1999) (Figure 8B)

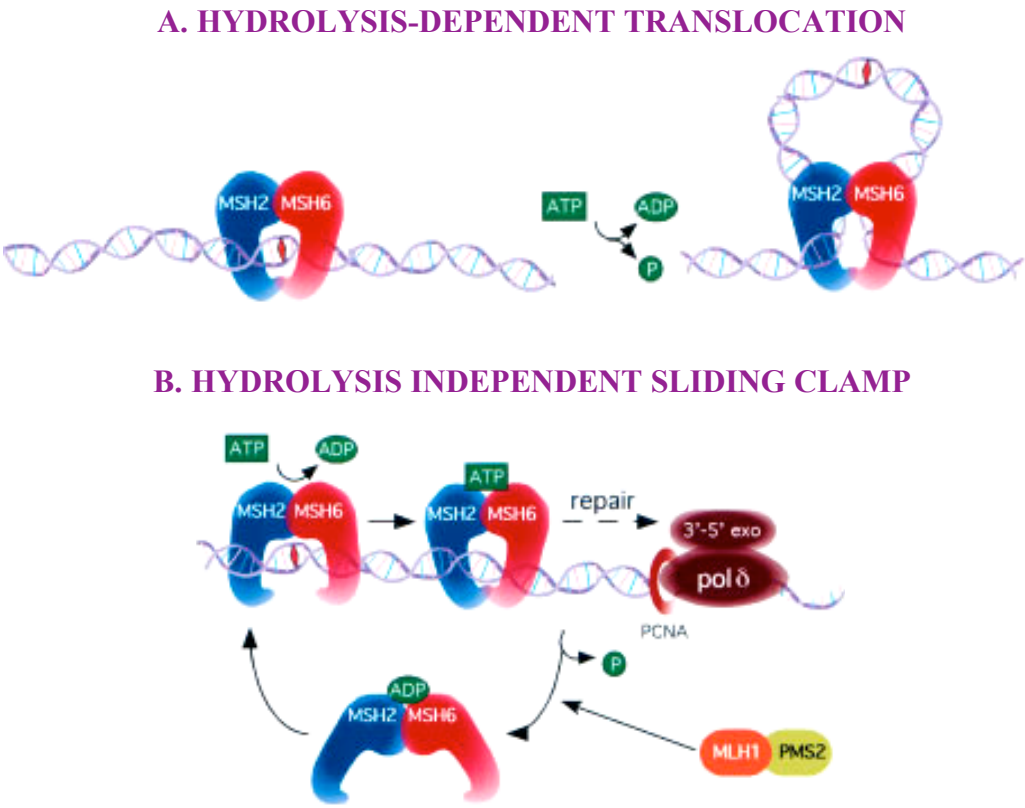


Figure 8. Models of Mutsα translocation.
A. In the 'ATP-dependent translocation' model, hMutSα upon mismatch binding moves away from the mismatch along the DNA, using the energy of ATP hydrolysis, thus linking mismatch recognition to the strand break or DNA terminus that directs excision to the newly-synthesized strand. B. Mismatch binding acts as an ADP-ATP exchange factor; the ATP bound MSH2-MSH6 complex becomes a 'sliding clamp' that diffuses freely in an ATP-hydrolysis-independent fashion along the DNA, signalling to additional components of the MMR machinery. Upon ATP hydrolysis that is perhaps stimulated by MLH1-PMS2, the MSH2-MSH6 complex is released from DNA (Bellacosa, 2001).

Upon binding to the mismatch, MutSα associates with another heterodimeric complex (MutLα), consisting of the MutL homologous mismatch repair proteins MLH1 and PMS2 (Li and Modrich, 1995). The excision of the DNA strand containing the mispaired base is thought to be catalysed by exonuclease I (Genschel et al., 2002) although it's still not clear whether this enzyme performs the excision *in vivo*. Resynthesis is most likely catalysed by Polδ (Longley et al., 1997).

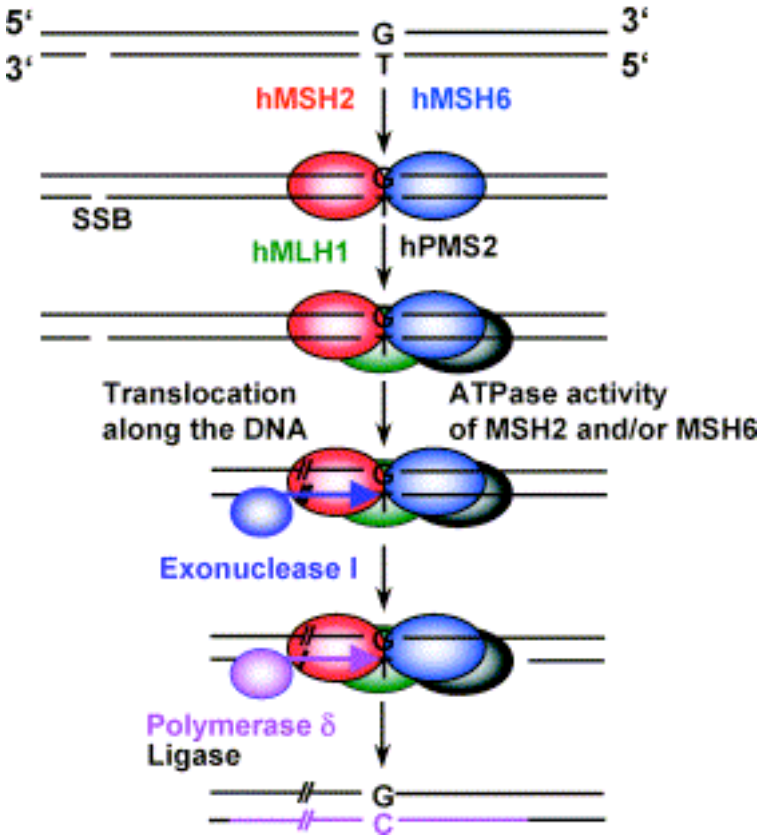


Figure 9. Model of human mismatch repair.
Recognition of DNA lesions occurs by MutSα (MSH2–MSH6). According to the molecular switch model, binding of MutSα–ADP triggers an ADP-ATP transition, stimulates intrinsic ATPase activity and provokes the formation of a hydrolysis-independent sliding clamp, followed by binding of the MutLα complex (MLH1–PMS2). According to the hydrolysis-driven translocation model, ATP hydrolysis induces translocation of MutSα along the DNA. After formation of a complex composed of MutSα and MutLα, excision is catalyzed by ExoI and repair synthesis by Polδ. Figure adopted from Christmann et al., 2003.

1.3 DOUBLE STRAND BREAK REPAIR

DNA double-strand breaks (DSBs) are highly potent inducers of chromosome aberrations and cell death. In higher eukaryotes, a single non-repaired DSB inactivating an essential gene can be sufficient for inducing cell death through apoptosis (Rich et al., 2000). Double-strand breaks are produced by reactive oxygen species, ionizing radiation and chemicals that generate reactive oxygen species. They are also a normal result of V(D)J recombination and immunoglobulin class-switching processes, but may also arise if a replication fork collides with an unrepaired DNA single-strand break (SSB), giving rise to a collapsed replication fork. In mammalian cells, these replication-fork-associated DSBs also trigger Homologous Recombination Repair (HRR) (Arnaudeau et al., 2001), even though they only have one free DNA end to initiate the repair. Recently, recombination has been implicated in the repair of stalled replication forks, which may also occur in the absence of detectable DSBs (Lundin et al., 2002). In this case, we know less about the substrates that initiate Homologous Recombination (HR). However, it has been shown in bacteria that nascent DNA strands may anneal and reverse stalled replication forks to form chicken-foot structures that may serve as a substrate for HR (McGlynn and Lloyd, 2002). Figure 10 shows DNA structures, which are substrates for DSB repair pathways in mammalian cells, and Table 4 summarizes products of the most common DNA damaging agents and pathways utilized to repair them.

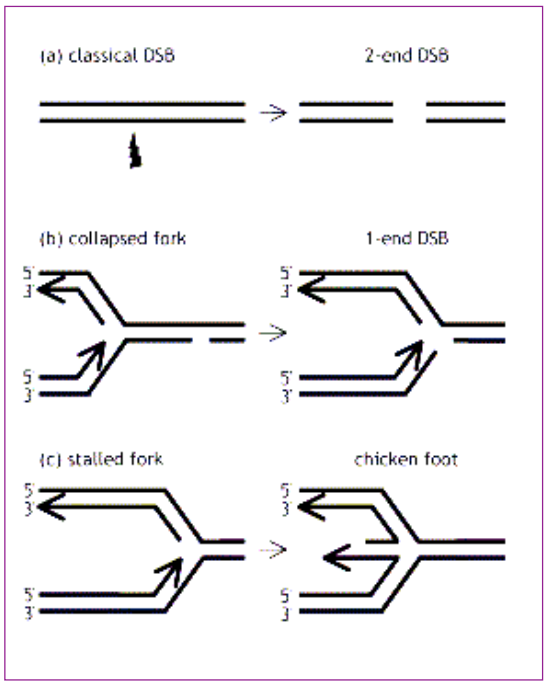


Figure 10. Structure of recombination substrates in mammalian cells.
(a) A classical DSB has two free DNA ends both of which may initiate recombination. (b) A persisting SSB may be converted into a DSB during replication that collapses the replication fork, leaving one free DNA end that is a substrate for recombination. (c) A replication fork may encounter roadblocks on the template DNA that stall the replication fork. Under such conditions the replication fork may reverse to form a chicken-foot intermediate that may serve as a substrate for recombination

Table 4. Summary of recombination substrates and products (Helleday, 2003)

Agents	*Recombinogenic lesion*	*Recombination pathway*	*Recombination product*
γ-rays, restriction endonucleases	Two-end DSB	NHEJ, HR	Deletion, gene conversion, duplication
Topoisomerase I inhibitors	One-end DSB	NHEJ, HR	SCE, deletions
Hydroxyurea, thymidine	Stalled fork	HR	Gene conversion, SCE

Repair of a classical double strand break (DSB) – two-end repair

There are two main pathways for repair of double-ended DSB, homologous recombination (HR) and non-homologous end-joining (NHEJ), which are error-free and error-prone, respectively. Both pathways are highly conserved throughout eukaryotic evolution, but their relative importance differs from one organism to another. In simple eukaryotes like yeast, HR is the main pathway, whereas in mammals the NHEJ pathway predominates (Cromie et al., 2001). The overlapping roles of HR and NHEJ in mammalian cells can be explained by the context in which DSB occurs. HR is favoured in S and G2 phase of the cell cycle when a sister chromatid is present and positioned close by, while NHEJ is predominant in G0 and G1 phases when the homologous chromosome is far away and the homology search would be almost impossible. This might also be a crucial factor in deciding whether a DSB is to be repaired by NHEJ or HR. Cells respond to exogenous and endogenous DSBs through a cascade of proteins ranging from sensors, which recognize the damage, through signal and mediator proteins, to a series of downstream effectors that induce cell-cycle arrests, complete repair by homologous or nonhomologous mechanisms, or alternatively trigger cell death by apoptosis (Jackson, 2002). Defects in almost any step of this response pathway can result in measurable alterations of DNA repair by HR and/or NHEJ. Mutations upstream in the cascade, before the decision is made whether a lesion is to be repaired by HR or NHEJ, can directly affect both principal recombinational repair pathways (Willers et al., 2004).

1.3.1 Non-homologous end joining (NHEJ)

The NHEJ system repairs DSBs without the requirement for sequence homology between the DNA ends and without the need for synapsis of the broken DNA with an undamaged partner DNA molecule (Jackson, 2002). It is generally agreed that at least in G0/G1 phases, the great majority of DSBs are rejoined by NHEJ (Takata et al., 1998). The prevalence of this error-prone mechanism over HR is presumably due to the difficulty of matching the damaged sequence to its intact copy on the homologous chromosome. Small changes in the sequence of DNA might be tolerated due to the fact that only a small proportion of mammalian genome is coding for genes and regulatory elements.

Although one might think that non-homologous end-joining would lead to the random joining of any two ends, this does not appear to be the case. Cells lacking crucial components of NHEJ undergo numerous chromosome translocations after DNA damage, but wild-type cells do not (Ferguson et al., 2000), implying that functional NHEJ in wild type cells must preferentially join correct ends of DSB. The components of NHEJ pathway are listed in Table 4. The core set of NHEJ proteins is conserved in all eukaryotic cells and includes DNA ligase IV, XRCC4, Ku70 and Ku80. Two additional proteins are present only in vertebrates, DNA-PKcs and Artemis. They evolved presumably to accomplish special functions of NHEJ that are required for V(D)J recombination, but are also important for general repair of DSB (Table 5). The basic mechanism of NHEJ is described in Figure 11.

Table 5. Genes and proteins important for NHEJ

Yeast gene	*Mammalian gene*	*Protein*
LIG4	LIG4	DNA ligase IV; cooperates with XRCC4 to ligate broken dsDNA molecules after their ends have been properly processed.
LIF1	XRCC4	XRCC4; cooperates with DNA ligase IV to ligate broken dsDNA molecules after their ends have been properly processed.
HDF2	XRCC5	Ku80; cooperates with Ku70 to bind DNA ends and recruit other proteins
HDF1	XRCC6	Ku70; cooperates with Ku80 to bind DNA ends and recruit other proteins
Not present in yeast	XRCC7	DNA-PKcs; protein kinase; activates Artemis
Not present in yeast	ARTEMIS	Artemis; nuclease regulated by DNA-PKcs; important for preparing DNA ends to make them ligatable

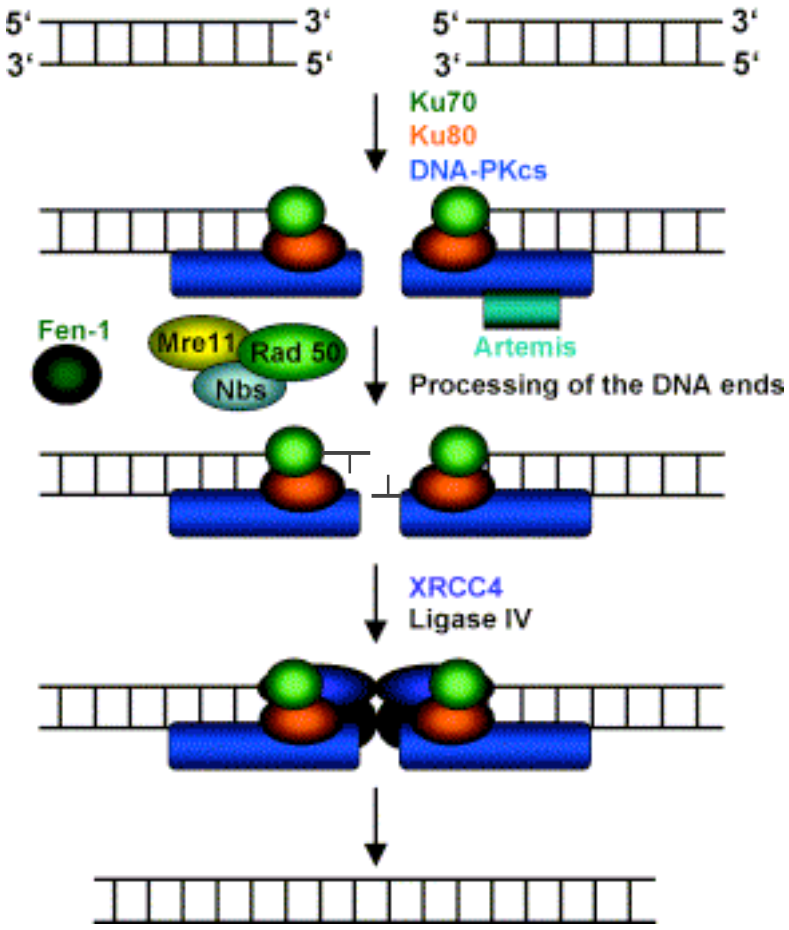


Figure 11. Mechanism of non-homologous end joining (NHEJ).
Recognition of and binding to damaged DNA ends is mediated by the Ku70–Ku80 complex. Thereafter, the Ku heterodimer binds to DNA–PKcs, forming the DNA–PK holoenzyme. DNA–PK activates XRCC4–ligase IV, which links the broken DNA ends together. Before re-ligation by XRCC4–ligase IV, the DNA ends are processed by the MRE11–Rad50–NBS1 complex, presumably involving FEN1 and Artemis. Figure adopted from (Christmann et al., 2003)

Central to NHEJ in organisms from yeast to man is the Ku protein, a heterodimer of two subunits, Ku70 (~69 kDa in man) (Reeves and Sthoeger, 1989) and Ku80 (~83 kDa in man; also known as Ku86) (Jeggo et al., 1992). The first step is the binding of a heterodimeric complex to the damaged DNA, thus protecting the DNA from exonuclease digestion. Following DNA binding, the Ku heterodimer associates with the catalytic subunit of DNA-dependent protein kinase (DNA–PKcs) (Hartley et al., 1995), thereby forming the active DNA–PK holoenzyme. DNA-PKcs is an ~465 kDa polypeptide, the C-terminal region of which has a homology to the catalytic domains of proteins of the phosphatidyl inositol 3-kinase-like (PIKK) family (Smith and Jackson, 1999). It has affinity for DNA ends and its activation appears to be triggered by its interaction with a single-stranded DNA region derived from a DSB (Hammarsten et al., 2000). Once bound to DNA DSBs, DNA-PK displays protein Ser/Thr kinase activity

with preference for the consensus sequence Ser/Thr-Gln (Kim et al., 1999). One of the targets of DNA–PKcs is XRCC4, which forms a stable complex with DNA ligase IV (Leber et al., 1998). The XRCC4–ligase IV complex binds to the ends of DNA molecules and links together duplex DNA molecules with complementary but non-ligatable ends. The XRCC4–ligase IV complex cannot directly re-ligate most DSBs generated by mutagenic agents—they have to be processed first because DSBs usually result in incompatible ends. Therefore, the rejoining typically requires nucleases to remove several nucleotides and polymerases to fill in gaps of several nucleotides. Some of these excess single-stranded regions require 5' nucleases, and others require 3' nucleases. Processing of DSBs is thought to be performed by the MRE11–RAD50–NBS1 complex (Maser et al., 1997), which displays exonuclease, endonuclease and helicase activity and removes excess DNA at 3′ flaps (Wu et al., 1999). However, the genetic evidence in mammals calls such a proposal into question. V(D)J recombination, which requires intact NHEJ for its joining phase, is normal in Nijmegen breakage syndrome (NBS)1-null cells or cells that are homozygous for shortened alleles of RAD50 (Harfst et al., 2000). A new gene, called Artemis, was identified on the basis of its mutation in patients with human severe combined immunodeficiency (SCID) (Moshous et al., 2001). It was shown that Artemis is a nuclease with a 5' exonuclease activity. It forms a complex with and can be phosphorylated by DNA-PKcs, upon which it acquires an endonuclease activity that opens hairpin loops, removes 5’ overhangs and shortens 3’ overhangs (Ma et al., 2002). One candidate responsible for removal of 5′ flaps is the flap endonuclease 1 (FEN1) (Wu et al., 1999).

After the ends of the DNA have been processed, XRCC4/Ligase IV complex can ligate the two duplex termini (Lee et al., 2003).

1.3.2 Homologous recombination

In contrast to NHEJ, few details of HR have been elucidated. For many years it was generally believed that homologous recombination was only a minor DSB repair pathway in mammalian cells, however recent work has shown that HR plays a substantial role in the repair of DSBs, especially those arising during replication. In order for this repair process to take place, homologous sequences in the form of sister chromatids, homologous chromosomes or DNA repeats are required, thus ensuring error-free repair. Another difference between NHEJ and HR is that HR is a very slow process, providing another explanation why NHEJ prevails in mammalian cells even though it's error-prone.

Table 6. Proteins involved in homologous recombination.

Protein	***Function***	
RAD51	Homologous pairing, recombinase	
RAD51B (RAD51L1)	RAD51 homologues	
RAD51C		Resolvase
RAD51D (RAD51D)		
XRCC2		
XRCC3		Resolvase
RAD52	Accessory factors for recombination	
RAD54L		
RAD54B		
BRCA1	Accessory factor for transcription and recombination, E3 Ubiquitin ligase	
BRCA2	Cooperation with RAD51, essential function	
DSS1 (SHFM1)	BRCA2 associated	
RAD50	ATPase in complex with MRE11A, NBS1	
MRE11	3'→5' exonuclease	
NBS1	Mutated in Nijmegen breakage syndrome, signalling	
MUS81	A structure-specific DNA nuclease	

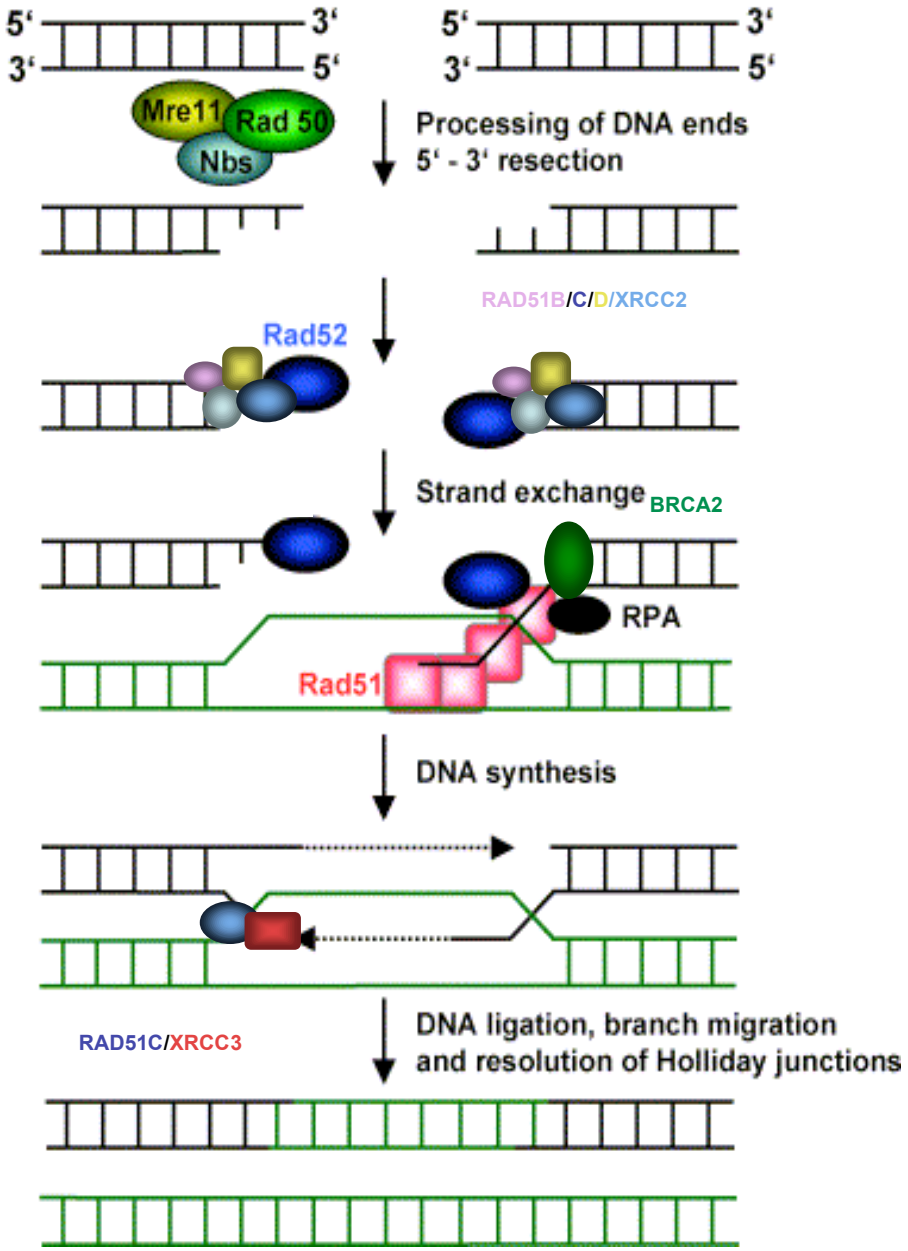


Figure 12. Principal steps and proteins involved in homologous recombination

HRR appears to involve a large number of proteins, including RAD51, RAD52, RAD54, BRCA1, BRCA2, the RAD51 paralogs RAD51B, C, D, XRCC2 and XRCC3, and the MRN complex (Table 6) (Wood et al., 2005). The first event believed to occur during HRR is resection of the DNA to yield single-strand overhangs. Based on analogy to yeast models, this resection is assumed to be 5'→3' and to involve the MRN complex; if so, other factors must also be involved as the MRE11 subunit itself has ssDNA endonuclease and 3'→5' exonuclease, but not 5'→3' exonuclease activity (Trujillo et al., 1998). The RAD50 subunit of MRN has ATPase activity that is believed to facilitate DNA unwinding, whereas the NBS1 subunit appears to be important for nuclear

transport and for transmitting signals from DNA damage sensors to MRN (Paull and Gellert, 1999). The resulting 3′ single-stranded DNA is thereafter bound by a heptameric ring complex formed by RAD52 (Stasiak et al., 2000), which protects against exonucleolytic digestion. RAD52 competes with the Ku complex for the binding to DNA ends. This may determine whether the DSB is repaired via the HR or the NHEJ pathway (Van Dyck et al., 1999). RAD52 interacts with RAD51 and RPA, stimulating DNA strand exchange activity of RAD51 (New et al., 1998). The human RAD51 protein is a homologue of the *E. coli* recombinase RecA. It forms nucleofilaments, binds single- and double-stranded DNA and promotes ATP-dependent interaction with a homologous region on an undamaged DNA molecule. RAD51 protein forms nucleoprotein complexes on ssDNA tails coated by RPA to initiate strand exchange. It catalyzes strand-exchange events with the complementary strand, in which the damaged DNA molecule invades the undamaged DNA duplex, displacing one strand as D-loop (Baumann and West, 1998). While the *RAD52* knockout mice are viable and healthy, a knockout of the *RAD51* gene is embryonic lethal, as are knockouts in other genes involved in HR (*RAD51B, RAD51D, XRCC2, RAD50, MRE11*, or *NBS1*), which implies that an intact HR pathway is vital (Helleday, 2003 and the references therein).

For invasion to occur, RAD51 must displace the RPA protein on the 3' ssDNA overhangs. RPA has much higher affinity for ssDNA than RAD51 and its replacement with RAD51 may be catalysed by the RAD51 paralogs (Sigurdsson et al., 2001). There are 5 paralogs of RAD51 in mammalian cells: RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3. They form two distinct complexes: BCDX2, composed of RAD51B, RAD51C, RAD51D and XRCC2, and CX3, composed of RAD51C and XRCC3 (Masson et al., 2001). In vertebrate cells, formation of DNA-damage-induced foci of RAD51 depends on the presence of RAD51 paralogs (Takata et al., 2001) thus indicating that loading of the RAD51 onto ssDNA requires functional paralogs. Interestingly, it was shown that the BCDX2 complex of RAD51 paralogs facilitates formation of RAD51 filaments on gapped DNA sequences (Masson et al., 2001). This is a very interesting observation, suggesting that strand invasion can be initiated without a free DNA end.

The proteins mutated in inherited forms of breast cancer, BRCA1 and BRCA2, are also involved at an early point of DSB repair. The BRCA2 protein may also play a role in loading RAD51 onto ssDNA (Powell and Kachnic, 2003), as it was shown that it has a preference for ss-ds DNA junctions and is required for recruitment of RAD51 and its localization to the sites of damage (Esashi et al., 2005). Although considerable indirect

evidence suggests that BRCA1 plays a direct role in HRR, the repair effects could be indirect through cell cycle control (Deming et al., 2001). Altogether, BRCA1 and BRCA2 are believed to be important at early points of HRR and perhaps coordinate repair with other cellular processes.

Strand invasion is further stimulated by the RAD54 protein. This protein forms negative supercoils in duplex DNA, which may increase accessibility for strand invasion (Tan et al., 2003).

Another group of proteins found mutated in patients with a rare recessive disease, Fanconi anaemia, has been implied to play a role in homologous recombination (West, 2003). It was shown that one of the *FANC* genes, *FANCD1* is in fact *BRCA2* (Howlett et al., 2002), thus linking the FANC protein complex to DNA repair (

Figure 13)

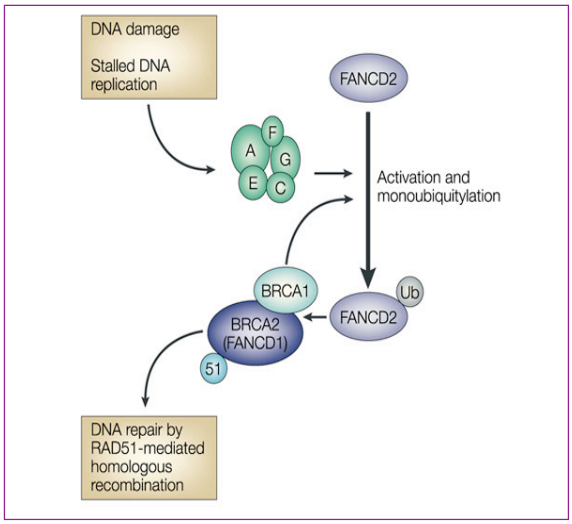
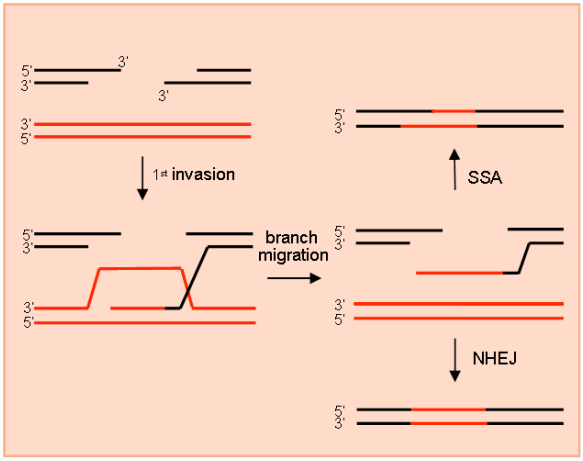


Figure 13. Fanconi anaemia complex.
The Fanconi anaemia (FA) proteins FANCA, FANCC, FANCE, FANCF and FANCG form a nuclear complex. In response to DNA damage, and during DNA replication, the complex can be activated. This results in the monoubiquitylation (Ub) of FANCD2 which requires BRCA1. The activated FANCD2 protein is then seen to colocalize with BRCA1 in nuclear foci, in which it might interact with other repair proteins. BRCA1 is known to interact with BRCA2 (which is also known as FANCD1), which, in turn, interacts with the RAD51 recombinase. The RAD51 protein has a direct role in DNA repair, thus completing the cycle. FANCB, which might also be related to BRCA2, is not shown (West, 2003).

Following the RAD51-catalysed invasion of the homologous DNA molecule during HR, DNA synthesis is initiated at the 3' end of the invading strand by a DNA polymerase. The synthesis is likely to proceed beyond the site of the original DSB. A Holliday Junction (HJ) that arose at the site of invasion, may branch migrate in either direction. If the HJ migrates in the direction of replication, it may reverse the invasion, leaving a DNA end that has been extended beyond the original DSB. This ssDNA end will share homology with the other end and may be repaired by a simple mechanism of Single Strand Annealing (SSA). However, synthesis-dependent SSA results in gene conversion, which is an error-free repair pathway, although it may result in loss of heterozygosity. Alternatively, the extended DNA end may be repaired by NHEJ. This synthesis-dependent NHEJ will result in tandem duplication at the site of the DSB. Tandem

duplications of this sort represent ~2% of all spontaneous gene mutations in the *hprt* gene in Chinese hamster cells showing that coupling of NHEJ and HR is important in the repair of spontaneous DSBs (Richardson and Jasin, 2000) (Figure 14).

Figure 14. First strand invasion in HR.
Strand invasion of a homologous DNA sequence may be initiated by one of the ssDNA ends. The invading strand is elongated past the site of breakage. Branch migration of the HJ may release the invading strand, unveiling homologous DNA sequences to the ssDNA overhang on the opposite DNA end. Synthesis-dependent SSA may use this homology in repair, which causes a gene conversion with no deletion. Synthesis-dependent NHEJ rejoins the extended DNA end without using the sequence homology. This will cause tandem duplications, giving a longer product than following synthesis-dependent SSA.



As an alternative to the release of the invading end, the second DNA end may invade the same homologous DNA molecule. This will result in a double HJ structure that may be resolved either by crossing over or non-crossing over (Figure 15).

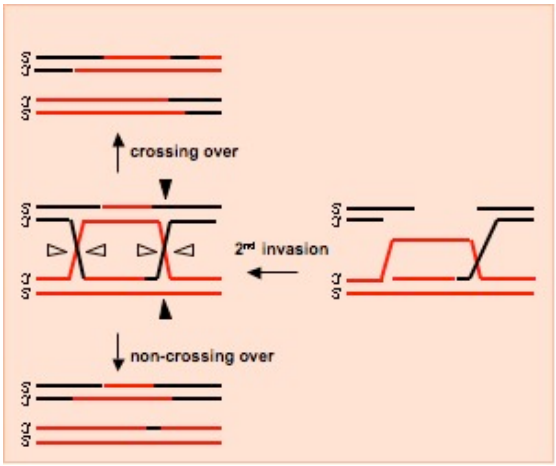


Figure 15. Second strand invasion in HR
Strand invasion by the second DNA end may occur if the invading strand is not released by branch migration; this causes a double HJ. In mammalian cells, these HJs are probably not resolved by crossing over (filled arrowheads), since cross-over products are suppressed in mitotic mammalian cells. Instead, the HJs may be resolved by non-crossing over (open arrowheads), causing a gene conversion event.

Cross-over events are probably rare in mammalian cells, since HRR using a sequence on another chromosome does not result in translocation (Richardson et al., 1998) and a SCE event has never been observed when analyzing the HR products following repair of an induced DSB (Johnson and Jasin, 2000).

Resolution of HJs in mammalian cells has been an enigma for many years. Resolvases from other organisms have been identified, like bacteriophage-T4 endonuclease VII, bacteriophage-T7 endonuclease I, pox virus A22, *E. coli* RuvC and RusA, archaeal Hjc

and Hje, and Saccharomyces cerevisiae mitochondrial Cce1 (Liu and West, 2004). It was shown that the resolvase complex in mammalian cells contains the recombination proteins RAD51C and XRCC3 (Liu et al., 2004) although the nuclease component of the complex is still not known.

Single-strand annealing in two-end repair

Single strand annealing can be considered as an intermediate pathway between NHEJ and HR. It requires a certain number of nucleotides of homology between 3' ends. If repeated sequences are uncovered within the resected 3' ssDNA overhangs, RAD52 and replication protein A (RPA) may initiate single-strand annealing (SSA) repair between the repeated sequences. The RAD52 protein binds the 3' ssDNA ends, whereas the RPA protein binds tightly to the 3' ssDNA overhangs. When these repeats are annealed, the regions between the repeats will be flipped out on either side. These are substrates for the ERCC1/XPF endonuclease that seems to play a role in SSA (Sargent et al., 2000). It has been suggested that SSA is a frequent repair event between repetitive sequences (Liang et al., 1998). The product formed by SSA is a deletion, making this pathway error-prone. Since a large proportion of mammalian genomes consists of repetitive sequences, e.g. Alu sequences, SSA may frequently be recruited in the repair of DSBs with two ends.

Recombination in repair of a collapsed replication fork—one end repair

The importance of the homologous recombination repair pathway is highlighted in the processes that attempt to repair collapsed replication forks. It has been difficult to establish the importance of RAD51 and other HR proteins in replication, as most HR knockout mice are embryonic lethal (Lim and Hasty, 1996). However, a conditional knockout RAD51-/- chicken cell line has been established. It has been reported to accumulate chromosome breaks during the first round of replication and arrests in the G2/M phase before entering apoptosis (Sonoda et al., 2001), suggesting that HR is involved in the repair of naturally occurring DSBs that arise during the S phase of the cell cycle. DSBs may be generated during replication by conversion of SSBs into DSBs, resulting in a collapsed replication fork. This collapsed fork may trigger break-induced replication (BIR) (Haber, 2000). The most important difference between a classical DSB and a replication-associated DSB at collapsed forks is that there is only one end to initiate HR (Figure 16). After the single-stranded gap in the template DNA strand has

been filled in, the free DNA end may invade this intact DNA molecule and resume replication. Following invasion, a single HJ will be left behind the replication fork. Since non-cross-over of a HJ is preferred resolution mechanism in mammalian mitotic recombination, the outcome of BIR will be a Sister Chromatid Exchange (SCE). The SCEs can be visualized since newly synthesised DNA is ligated to template DNA. Following a second mitosis, the break site may be clearly scored as a SCE by cytological methods (see Materials and Methods).

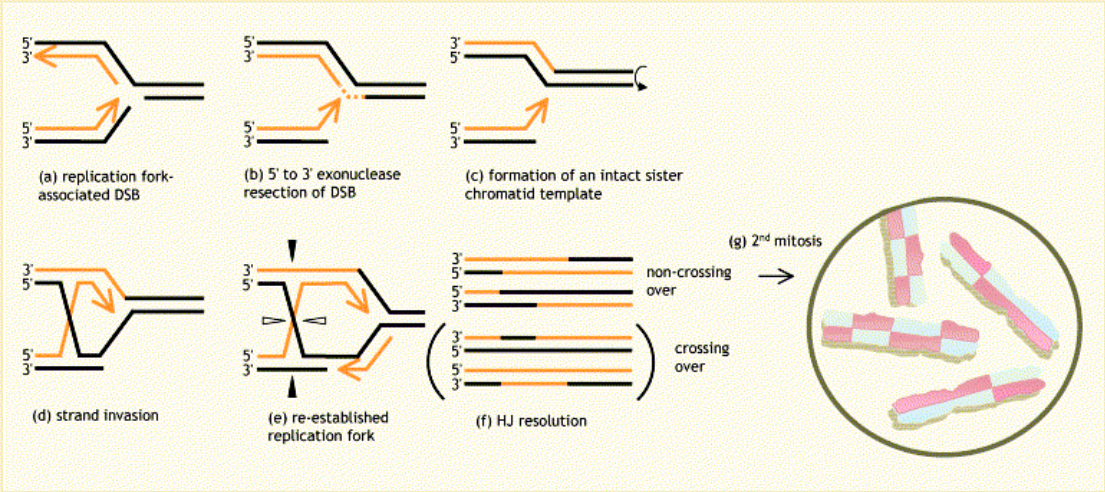


Figure 16. HRR of a collapsed replication fork, i.e. one-end repair.
(a) A replication fork-associated DSB has one free end to initiate exonuclease resection. (b) The 3' ssDNA overhang is coated with RAD51 and other HR proteins involved in strand invasion. (c) The single-strand gap on the template DNA will be filled in advance of (d) strand invasion. Leading strand synthesis may continue on the invaded template DNA, and a replication fork is re-established (e). This re-established replication fork will have swapped the leading and lagging strands; compare (a) and (e). (f) A single HJ is left behind the replication fork, this is probably resolved by non-crossing over since crossing over is an unlikely event. Since template DNA and newly synthesised DNA are fused following non-crossing over, SCE will be visualised following a second mitosis (g); open arrowheads designate non-crossing over and filled arrowheads indicate crossing over (Helleday, 2003).

Recombination at stalled replication forks

It's clear that stalled replication forks are probably the most frequent substrates for recombinational repair in mammalian cells (Lundin et al., 2002). In addition to the fact that a deficiency in HR is embryonic lethal in mice (Deans et al., 2000; Douglas L. Pittman, 2000; Tsuzuki et al., 1996) Chinese hamster cells deficient in HR show delayed progress through the cell cycle (Fuller and Painter, 1988), hypersensitivity to agents that stall replication (Lundin et al., 2002) and chromosome instability (Cui et al., 1999). Furthermore, agents that inhibit replication are potent inducers of HR and RAD51 focus formation (Saintigny et al., 2001). RAD51 foci have been shown to form in postreplicative DNA and at sites of stalled replication forks (Sengupta et al., 2003). Elaborate systems for the repair of broken replication forks exist in all eukaryotic cells. It has been suggested that about 10 replication forks collapse or arrest in human cells per replication cycle (Cox, 2002; Haber, 1999)

Emerging evidence from bacteria shows that stalled replication forks may reverse to form an intermediate "chicken-foot" structure that may be repaired by trans-lesion synthesis or recombination (Michel et al., 2001; Michel et al., 2004). It is possible that stalled replication forks may also reverse to form chicken-foot structures in mammalian cells, although there is no direct evidence thus far. Some mammalian cell lines treated with hydroxyurea accumulate DSBs at or near replication forks (Bianchi et al., 1986; Lundin et al., 2002) and in this case both NHEJ and HR participate in the repair of broken replication forks. In the case of thymidine, which was reported not to cause double strand breaks, HR alone is involved in the repair (Lundin et al., 2002), suggesting that NHEJ is only involved in the repair of blocked replication forks that have been processed into a DSB. More importantly, it indicates that HR repairs lesions at stalled replication forks that do not appear as detectable DSBs. The conclusion from these and other experiments is that HR repairs a broader spectrum of lesions that occur at stalled replication forks, while NHEJ only repairs DSBs (Lundin et al., 2003).

1.4 DNA DAMAGE RESPONSE

The fast response to DNA damage is necessary in order to prevent transmission of damaged DNA to daughter cells, thus avoiding dangerous mutations. This fast response cannot rely on transcriptional activation, at least not in the time that follows detection of the damage. Therefore, two mechanisms may be in play: cascades of posttranslational modifications, which change the affinities of proteins for their substrates without the need to synthesize new proteins, and/or recruitment of repair proteins to the sites of damage, thus creating microenvironment that favours repair. In most instances, these two mechanisms are interconnected and sometimes it's difficult to distinguish effects of DNA damage signalling from DNA repair.

1.4.1 DNA damage signalling and checkpoints

DNA damage checkpoints are biochemical pathways that delay or arrest cell cycle progression in response to DNA damage. Besides defining the specific time point in the cell cycle when the DNA is "checked", the term has been applied to the entire ensemble of cellular responses to DNA damage, including the arrest of cell cycle progression, induction of DNA repair genes, and apoptosis. It should be noted though, that DNA repair pathways are functional in the absence of damage-induced cell-cycle arrest, and that apoptosis can occur independently of the cell-cycle arrest machinery. Generally speaking, DNA damage checkpoints are events that slow or arrest cell-cycle progression in response to DNA damage in order to provide more time for the repair machinery to revert the damage.

All eukaryotic cells have four phases within the cell cycle, G1, S, G2, and M, and one outside, G0. In mammalian somatic cells, the phases are well defined and represent stages in the life of a cell in which distinct biochemical reactions take place. In an unperturbed cell cycle, the transition points G1/S and G2/M, as well as S-phase progression, are tightly controlled, and the same proteins involved in regulating the progression through the cell cycle are also involved in checkpoint responses. (Sancar et al., 2004). The DNA damage checkpoints, like other signal transduction pathways, conceptually have three components: sensors, signal transducers, and effectors (Nyberg et al., 2002). Although the G1/S, intra-S, and the G2/M checkpoints are distinct, the damage sensor molecules that activate the various checkpoints appear to either be shared

by all three pathways or to play a primary sensor role in one pathway and a back-up role in the others. Similarly, the signal-transducing molecules, which are protein kinases and phosphatases, are shared by the different checkpoints (Zhou and Elledge, 2000). The effector components (proteins that inhibit phase transition) of the checkpoints are what gives the checkpoints their unique identities.

Recognition of aberrant DNA is accomplished by a group of phosphatidylinositol-3-kinase-like kinases. These kinases are ATM (Ataxia Telangiectasia Mutated, (Savitsky et al., 1995)), ATR (Ataxia Telangiectasia and Rad3 Related) and the catalytic subunit of DNA–PK (Hartley et al., 1995), Rad17-RFC and Rad9-Rad1-Hus1 complexes.

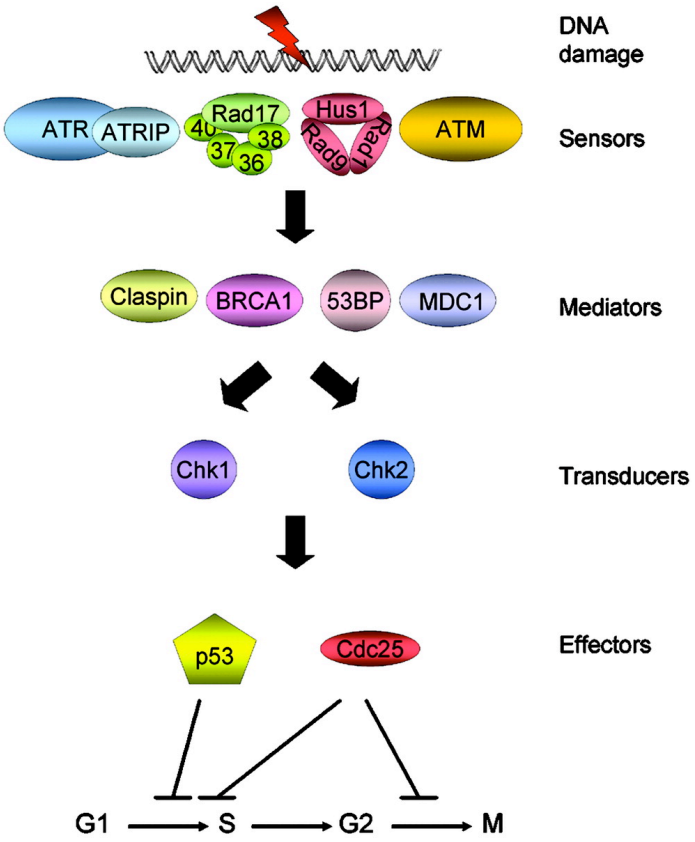


Figure 17. Components of the DNA damage checkpoints in human cells.
The damage is detected by sensors that, with the aid of mediators, transduce the signal to transducers, which then, activate or inactivate other proteins (effectors) that directly participate in inhibiting the G1/S transition, S-phase progression, or the G2/M transition (Sancar et al., 2004).

ATM is a 350-kDa oligomeric protein found mutated in ATM (ataxia telangiectasia mutated), a condition primarily characterized by cerebellar degeneration, immunodeficiency, genome instability, clinical radiosensitivity and cancer predisposition (Shiloh, 1997). Upon exposure of cells to ionizing radiation, ATM phosphorylates many proteins, including Chk2, p53 (Canman et al., 1998), NBS1 (Lim et al., 2000), BRCA1

(Cortez et al., 1999) and itself (Bakkenist and Kastan, 2003) at serines and threonines in the SQ or TQ sequence context. Autophosphorylation of ATM converts the oligomer into monomers, which appear to be the active form of the enzyme for the checkpoint response (Bakkenist and Kastan, 2003).

ATR was discovered in the human genome database as a gene with sequence homology to ATM and SpRad3, hence the name ATR (ATM and Rad3 related) (Cimprich et al., 1996). The gene encodes a protein of 303 kDa with a C-terminal kinase domain and regions of homology to other PIKK family members. Knockout of ATR in mice results in embryonic lethality and a partial loss of ATR activity in humans has been associated with the human autosomal recessive disorder Seckel syndrome, which shares similarities with AT (O'Driscoll and Jeggo, 2003). Complete deficiency of ATR is lethal. ATR, like ATM, is a protein kinase with specificity for S and T residues in SQ/TQ sequences and it phosphorylates essentially all proteins that are phosphorylated by ATM. In contrast to ATM however, ATR is activated *in vivo* by UV light rather than by ionizing radiation, and it is the main PIKK family member that initiates signal transduction following UV irradiation. Thus, at present it appears that ATM is a sensor and transducer responding to double-strand breaks, and ATR serves an analogous role for base damage, at least from UV irradiation.

DNA-PK is a heterotrimer of a 450-kDa catalytic subunit (DNA-PKcs) and a dimer of Ku70 and Ku80. The Ku70/Ku80 dimer binds to DNA ends and recruits DNA-PKcs, which then becomes activated as a DNA-dependent protein kinase (Gottlieb and Jackson, 1993).

The Rad17-RFC complex is a checkpoint-specific structural homolog of the replication factor C (RFC). The replicative form of RFC is a heteropentamer composed of p140, p40, p38, p37, and p36. In Rad17-RFC, the p140 subunit is replaced by the 75-kDa Rad17 protein (Lindsey-Boltz et al., 2001). The 9-1-1 (Rad9-Rad1-Hus1) complex is the checkpoint counterpart of PCNA, a homotrimer with a ring-like structure. Although the Rad9, Rad1 and Hus1 proteins have little sequence homology to PCNA, or to one another, molecular modelling suggested that they might form a PCNA-like structure (Venclovas and Thelen, 2000).

In humans, there are two kinases, Chk1 and Chk2, with a strictly signal transduction function in cell cycle regulation and checkpoint responses (Melo and Toczyski, 2002). Both Chk1 and Chk2 are S/T kinases with limited substrate specificities. In mammalian cells, the double-strand break signal sensed by ATM is transduced by Chk2 (Matsuoka et

al., 2000) and the UV-damage signal sensed by ATR is transduced by Chk1 (Zhao and Piwnica-Worms, 2001). However, there is some overlap between the functions of the two proteins.

Three phosphotyrosine phosphatases, Cdc25A, -B, and -C, downstream targets of DNA damage transducers, dephosphorylate the cyclin-dependent kinases that act on proteins directly involved in cell-cycle transitions. Phosphorylation inactivates the Cdc25 proteins by excluding them from the nucleus, by causing proteolytic degradation, or both. Unphosphorylated Cdc25 proteins promote the G1/S transition by dephosphorylating Cdk2 and promote the G2/M transition by dephosphorylating Cdc2 phosphotyrosine (Bartek and Lukas, 2001).

The G1/S checkpoint

The G1/S checkpoint prevents cells from entering the S phase in the presence of DNA damage by inhibiting the initiation of replication. If the DNA damage is double-strand breaks caused by ionizing radiation or radiomimetic agents, ATM is activated and phosphorylates many target molecules, such as p53 and Chk2. These phosphorylations result in the activation of two signal transduction pathways, one to initiate and one to maintain the G1/S arrest (Bartek and Lukas, 2001).

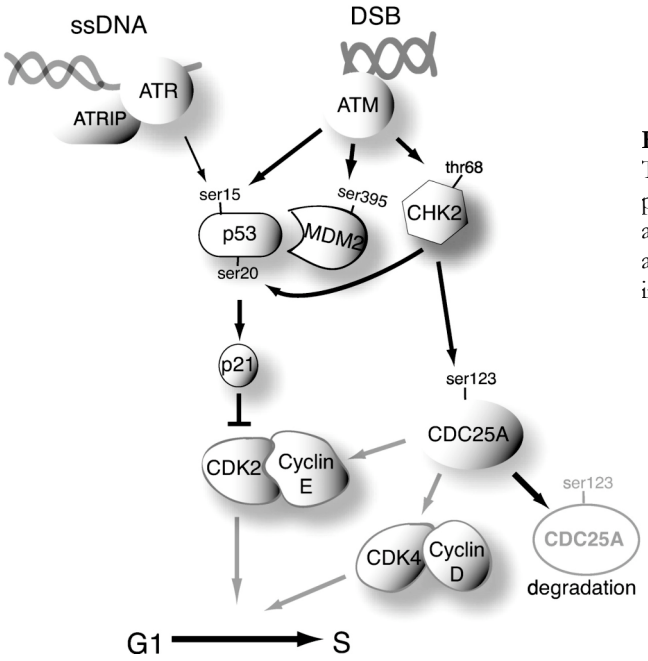


Figure 18. G1/S checkpoint.
The G1 checkpoint in mammalian cells primarily functions to block Cdk2-cyclin E activity. This is achieved by stabilizing p53 and degrading Cdc25A to maintain Cdk2 inhibitory phosphorylation.

The reaction that initiates the G1/S arrest is phosphorylation of Chk2, which in turn phosphorylates Cdc25A phosphatase, causing its inactivation by nuclear exclusion and

ubiquitin-mediated proteolytic degradation. If the DNA damage is induced by UV light or UV-mimetic agents, the signal is sensed by ATR, Rad17-RFC, and the 9-1-1 complexes, leading to phosphorylation of Chk1 by ATR. The activated Chk1 then phosphorylates Cdc25A, leading to a G1 arrest. Whether the initial arrest is caused by the ATM-Chk2-Cdc25A pathway or the ATR-Chk1-Cdc25A pathway, this rapid response is followed by the p53-mediated maintenance of G1/S arrest, which becomes fully operational several hours after the detection of DNA damage (Bartek and Lukas, 2001; Nyberg et al., 2002) (Figure 18).

G2/M checkpoint

The G2/M checkpoint prevents cells from undergoing mitosis in the presence of DNA damage. Depending on the type of DNA damage, the ATM-Chk2-Cdc25 signal transduction pathway and/or the ATR-Chk1-Cdc25 pathway is activated to arrest the cell cycle following DNA damage in G2. These two pathways converge in phosphorylation of CDC25C, thus blocking its phosphates activity required to dephosporylate Cdc2 in order to make it active and allow entry into mitosis (Figure 19).

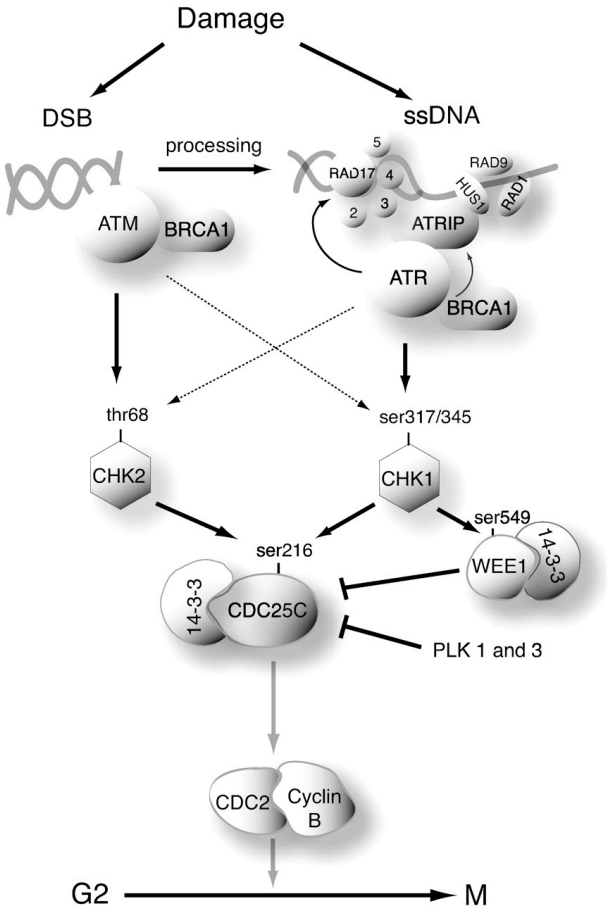


Figure 19. The G2 checkpoint
In mammalian cells it primarily functions to block the Cdc2-cyclin B activity. The common means of maintaining Cdc2 inhibitory phosphorylation is by blocking the Cdc25C phosphatase activity, namely by promoting its association with 14-3-3-proteins.

1.4.2 Nuclear dynamics of repair proteins

It has been hypothesised that the spatial organisation of chromatin and nuclear protein complexes is essential for the proper regulation of processes that take place in the nucleus. The nucleus, in this view, consists of a number of immobile protein complexes, often referred to as factories in which transcription, replication, repair or RNA processing take place (Cardoso et al., 1999). Enzymes required for function are actively recruited to these factories, and DNA is reeled through the fixed structures as it is being processed. In sharp contrast to this concept of highly compartmentalised nuclear activity stands the view that the nucleus is far less organised in a structural sense and that nuclear processes are regulated by freely diffusing proteins. Microscopically visible protein/DNA/RNA structures are formed, in this view, as a result of protein activity, and they do not represent prerequisites for proper function (Lewis and Tollervey, 2000).
Answers to question of protein mobility and assembly of mutiprotein complexes in the nuclei of mammalian cells started to get answered only after GFP technology became available and expression of GFP-tagged proteins became possible. It has been shown using FRAP (Fluorescence Recovery After Photobleaching), that dextrans up to a molecular weight of 500 kDa diffuse freely through the nucleus (Houtsmuller and Vermeulen, 2001). In addition, free GFP homogeneously stains the living nucleus and nucleotide excision repair proteins diffuse with rates consistent with their molecular weight, and show a homogeneous distribution throughout the nucleus (Houtsmuller et al., 1999), indicating that most of the proteins find their way through the interchromatin compartments easily. It's possible that those molecules travel through interchromatin space only, and that light microscopic resolution is not high enough to reveal this because the interchromatin channels are too small. However, it seems unlikely that repair factors only have access to the interchromatin space, while DNA damage is distributed randomly throughout the entire genome and also in the interior of condensed heterochromatin areas. DNA itself could move upon damage induction, bringing lesions to the surface of chromatin territories. However, it has been shown with FRAP that chromatin itself is quite immobile (Abney et al., 1997).

In vivo studies of DNA repair proteins have shown that in non-damaged cells, most of the repair proteins have uniform distribution in the nucleoplasm. Relocalization of the proteins and formation of so-called foci has been a hallmark of DNA damage.

Formation of foci, or local accumulation of a protein, is also noticed in undamaged cells, but is associated with normal DNA metabolism, as shown by PCNA staining of normal cycling cells (Essers et al., 2005). Colocalization studies of DNA repair proteins have been a useful tool in linking biochemical data and in vivo observations. DNA damaged induced foci are thought to be markers of damaged DNA as was shown by experiments using local UV irradiation through filter pores and following subsequent recruitment of the NER proteins (Volker et al., 2001). With these experiments it became clear that proteins engaged in repair of certain lesions are not permanently "trapped" in a position inside the nuclear matrix as was thought before, but rather move freely and accumulate at the sites of damage. Also, it's important to notice that once inside the focus, repair proteins are not bound permanently but diffuse freely, assumingly as soon as they dissociate from the damaged DNA (Schermelleh et al., 2005).

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3 AIMS AND SCOPE

The principal role of mismatch repair is repairing mismatches that arise during DNA replication and escape the proofreading activity of replicative polymerases. Besides this well characterized role of MMR, its involvement in other DNA transactions has been reported. Studies of the mechanism of MMR have been mostly limited to biochemical and *in vitro* studies due to the fact that induction of mismatches in the genomic DNA is experimentally impossible. In order to study the mechanism of MMR *in vivo*, I used the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), whose cytotoxicity is known to be mediated by MMR. Activation of MMR after MNNG treatment is thought to be due to formation of ^{6me}G residues that are recognized by the polymerase as adenine and therefore have tendency to base pair with thymine. Mismatches of this kind are permanent, because ^{6me}G persists in the template strand that MMR processing cannot affect. But details of the action of MMR proteins are not known and the hypothesis trying to explain its role in the cytotoxicity of MNNG failed to be proven. What is the mechanism by which MMR addresses MNNG induced damage, and moreover, what are the pathways involved in the further processing of the damage, have not been studied so far.

The aim of my study was to characterize the *in vivo* role of MMR proteins, especially in mediating cytotoxicity of MNNG. Consequence of MNNG treatment is activation of cell cycle checkpoint on one side, and an attempt to repair the DNA intermediates on the other hand. My interest was to characterize DNA repair pathways that are activated in MMR dependent manner after MNNG treatment and to elucidate why these repair attempts fail.

4 RESULTS

Short summaries and reprints of publications describing the results obtained during my PhD work.

4.1 Methylation-induced G_2/M arrest requires a full complement of the mismatch repair protein hMLH1

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This publication describes the construction and characterization of the 293T Lα cell line, in which expression of hMLH1 can be regulated by doxycycline. Availability of such a cell line presents a major achievement in the field, because previous pairs of MMR-proficient and -deficient cell lines were continuously drifting apart in their genetic makeover due to the mutator fenotype of MMR deficiency. Human embryonic kidney 293T cells expressing high amounts of hMLH1 were MMR-proficient and arrested at the G_2/M cell cycle checkpoint following treatment with the DNA methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), while cells not expressing hMLH1 displayed a MMR defect and failed to arrest upon MNNG treatment. Interestingly, MMR proficiency was restored even at low hMLH1 concentrations, while checkpoint activation required a full complement of hMLH1. The observed defect may be relevant to cellular transformation and cancer.

Methylation-induced G_2/M arrest requires a full complement of the mismatch repair protein hMLH1

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The mismatch repair (MMR) gene *hMLH1* is mutated in ~50% of hereditary non-polyposis colon cancers and transcriptionally silenced in ~25% of sporadic tumours of the right colon. Cells lacking hMLH1 display microsatellite instability and resistance to killing by methylating agents. In an attempt to study the phenotypic effects of hMLH1 downregulation in greater detail, we designed an isogenic system, in which hMLH1 expression is regulated by doxycycline. We now report that human embryonic kidney 293T cells expressing high amounts of hMLH1 were MMR-proficient and arrested at the G_2/M cell cycle checkpoint following treatment with the DNA methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), while cells not expressing hMLH1 displayed a MMR defect and failed to arrest upon MNNG treatment. Interestingly, MMR proficiency was restored even at low hMLH1 concentrations, while checkpoint activation required a full complement of hMLH1. In the MMR-proficient cells, activation of the MNNG-induced G_2/M checkpoint was accompanied by phosphorylation of p53, but the cell death pathway was p53 independent, as the latter polypeptide is functionally inactivated in these cells by SV40 large T antigen.
Keywords: cell cycle checkpoint/hMLH1/methylating agent/mismatch repair/TetOff

Introduction

Mutations in mismatch repair (*MMR*) genes, predominantly *hMSH2* and *hMLH1*, segregate with hereditary non-polyposis colon cancer (HNPCC). Inheritance of a single mutated allele of a *MMR* gene predisposes to precocious cancers of the colon, endometrium and ovary. Analysis of HNPCC tumour cells showed that repeated sequence elements (microsatellites) in their genomic DNA are frequently mutated (for a review see Peltomaki, 2001). As microsatellite instability (MSI) is a hallmark of defective MMR in all organisms tested to date, and has been shown to be present in all tumour cell lines that have lost both alleles of *hMSH2* or *hMLH1* (Boyer *et al*., 1995), it is assumed that the wild type alleles of the respective *MMR* genes in cells of HNPCC tumours have been lost or inactivated by mutation. But mutations in *MMR* genes are not an absolute prerequisite for MSI. In recent years, a number of sporadic colon tumours and tumour cell lines displaying MSI have been described that are MMR-deficient due to silencing of the *hMLH1* promoter by hypermethylation (reviewed in Esteller, 2002).

Once both *MMR* gene alleles have been inactivated, the cell's propensity towards acquiring mutations increases, especially in genes carrying microsatellite repeats. Should the mutated genes be involved in the control of cell proliferation, the mutator cell in, for example, the colonic epithelium would be able to divide in an uncontrolled manner and thus give rise to an adenomatous polyp. As the cells in this benign growth acquire further mutations with subsequent cell divisions, the adenoma would rapidly become transformed into a carcinoma. That such a path to transformation can be followed *in vivo* was demonstrated when numerous HNPCC colon cancers were shown to carry frameshift mutations in a run of 10 adenines within the coding sequence of the transforming growth factor β receptor type II (*TGFβRII*) gene, as well as in other genes involved in growth control or apoptosis (reviewed in Markowitz *et al*., 2002). Further support for this hypothesis comes from the finding that adenomas of HNPCC kindred transform to carcinomas with a much higher frequency than those associated with sporadic disease (Kinzler and Vogelstein, 1998), presumably due to a more rapid acquisition of transforming mutations.

The above findings help explain how the loss of MMR might accelerate cellular transformation and tumour progression. What is unclear to date, however, is whether the transformation process begins only following the inactivation of both *MMR* gene alleles, or whether it commences already at the stage when only one allele is affected or when the expression of the given *MMR* gene is only attenuated, rather than shut off, such as might be the case in cells where the *hMLH1* promoter is only partially methylated. The notion that a reduction in MMR protein levels might promote tumorigenesis originates in studies with $Msh2^{+/-}$ mice. Although the $Msh2^{+/-}$ embryonic stem cells were apparently normal in terms of their MMR capacity as measured by MSI (de Wind *et al*., 1995), the heterozygous animals were cancer prone, and presented with tumours that often still contained the wild-type *Msh2* allele (de Wind *et al*., 1998). The propensity of the *MMR* heterozygous cells to transformation would thus appear to be linked to a process distinct from the correction of replication errors. What might the nature of these processes be?

In recent years, MMR defects have been linked to several other phenomena, such as transcription-coupled repair and recombination—both mitotic and meiotic (reviewed in Harfe and Jinks-Robertson, 2000). In

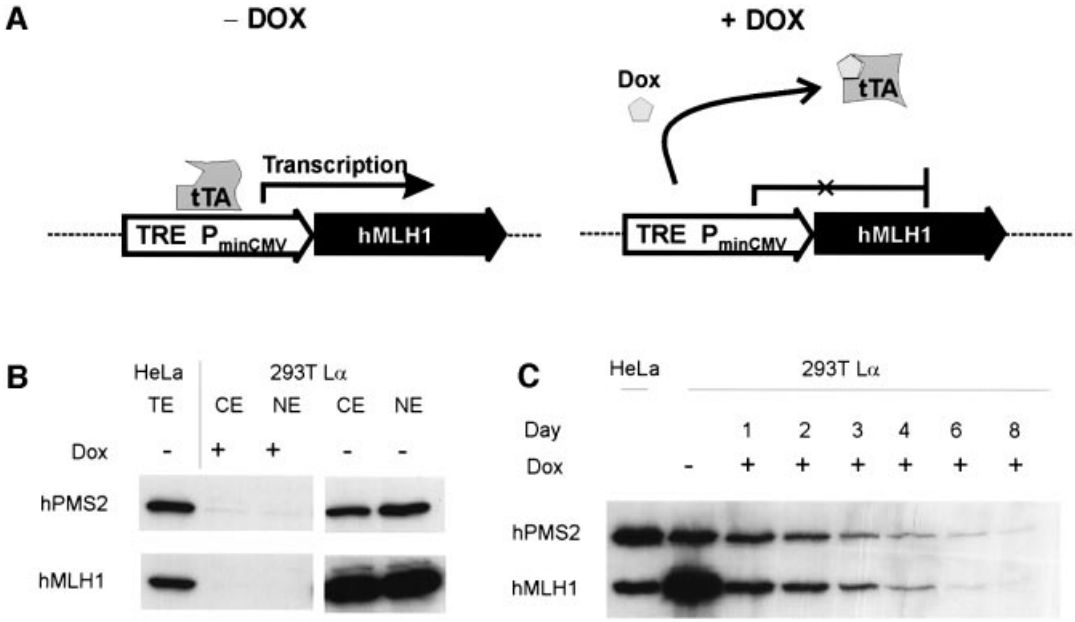


Fig. 1. Inducible *hMLH1* expression in 293T Lα cells. (**A**) In the Tet-Off system, hMLH1 is expressed in the absence of Dox, because the tTA factor binds to the promoter of the expression vector and thus activates transcription. Addition of Dox to the culture medium causes a conformational change in tTA, which leads to its dissociation from the promoter and thus to an inactivation of *hMLH1* transcription. (**B**) Western blot analysis of cytoplasmic (CE) and nuclear (NE) extracts of cells cultured in the absence (–) or presence (+) of 50 ng/ml Dox. hMLH1 and hPMS2 were visualized using anti-hMLH1 or anti-hPMS2 antibodies as described in Materials and methods. Total extract (TE) of MMR proficient HeLa cells was used as a positive control. (**C**) Stability of hMutLα. The cells were cultured without Dox (–) to induce maximal hMLH1 expression. Following the addition of 50 ng/ml Dox (+), total cell extracts were isolated after 1, 2, 3, 4, 6 and 8 days. Western blot analysis was performed using anti-hMLH1 and anti-hPMS2 antibodies as in (B).

addition, the MMR system was implicated in activation of cell cycle checkpoints and apoptosis, as witnessed by the increased resistance of MMR-deficient cells to the methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) or cisplatin (reviewed in Bellacosa, 2001). Thus, while $MMR^{+/-}$ cells, or cells expressing low amounts of MMR proteins, may not display a mutator phenotype, they might have at least a partial defect in one of the above processes, specifically in the DNA damage signalling pathway, which we judged to be of the greatest relevance to cancer. We wanted to study these processes in detail, but we lacked isogenic cells expressing varying amounts of MMR proteins. Cells in which the MMR defect was corrected either by transfer of a chromosome carrying a single wild-type copy of the mutated *MMR* gene (Koi *et al*., 1994) or its cDNA (Risinger *et al*., 1998; Buermeyer *et al*., 1999; Lettieri *et al*., 1999; Claij and Te Riele, 2002) were unsuitable for our studies, because they express similar or even higher amounts of the complementing MMR proteins than MMR-proficient controls. Thus, in order to be able to study the phenotypic consequences associated with reduced levels of MMR proteins, we had to generate a new line, preferably of epithelial origin, in which the expression of a selected *MMR* gene could be regulated. We now describe the construction and characterization of a line in which the expression of *hMLH1* can be tightly regulated by doxycycline with the help of the TetOff system.

Results

Construction of cells with inducible hMLH1 expression

The human embryonic kidney cell line 293T is MMR deficient, because the *hMLH1* gene in these cells is epigenetically silenced by promoter hypermethylation (Trojan *et al*., 2002). We set out to correct its MMR defect through the expression of exogenous hMLH1 using the TetOff expression system, which can be tightly regulated. We first generated the 293T-TetOff cell line by stable transfection of the 293T cells with a DNA vector encoding the tetracycline-controlled transactivator (tTA). In the second step, we stably transfected the 293T-TetOff cells with a vector carrying the *hMLH1* cDNA under the control of the tetracycline response element (TRE), thus creating 293T Lα cells. In the absence of tetracycline, or its more stable analogue doxycycline (Dox), the tTA protein binds to the TRE and activates transcription of *hMLH1*; conversely, addition of the drug induces a conformational change in tTA, which loses its ability to bind DNA and the transcription of *hMLH1* is thus turned off (Figure 1A). During the initial screening, we used Dox at a concentration of 2 μg/ml, as recommended by the manufacturer, but later we found that a concentration of 50 ng/ml was sufficient to turn off the expression of hMLH1 below the limit of detection by western blotting (see below).

In vivo, hMLH1 interacts with hPMS2 to form the heterodimer hMutLα, which is essential for MMR. Our previous studies have shown that hPMS2 is unstable in the absence of its cognate partner (Räschle *et al*., 1999). Indeed, no hMLH1 could be detected in the extracts of 293T cells, and hPMS2 was hardly detectable (Trojan *et al*., 2002). A similar situation also existed in our 293T Lα clone grown in the presence of Dox, i.e. under conditions where the *hMLH1* promoter is shut off (Figure 1B). However, expression of hMLH1 brought about hPMS2 stabilization through the formation of hMutLα, such that the levels of the latter protein were

comparable to those seen in extracts of MMR-proficient cell lines (Figure 1B).

The expression of hMLH1 in the 293T Lα cells grown in the absence of Dox was substantially higher than in any MMR-proficient cell line tested by us to date (Figure 1B; data not shown). Interestingly, this overexpression did not appear to be toxic to the cells: we detected no increase in the rates of apoptosis, as described for cells microinjected with expression vectors encoding hMSH2 and hMLH1 (Zhang *et al*., 1999). Moreover, cells grown in the absence or presence of Dox divided roughly once every 24 h (data not shown), unlike HCT116 and SNU-1 cells, in which the stable expression of hMLH1 was reported to result in substantially slower growth rates (Shin *et al*., 1998). When the expression of the transgene was turned off by the addition of Dox, the hMLH1 and hPMS2 proteins were present in the cell extracts in a 1:1 ratio only 24 h later (Figure 1C) and decayed with similar kinetics. This experiment showed that hMutLα is extremely stable, as it was detectable in the extracts of 293T Lα cells even 6 days after the expression of hMLH1 was shut off.

In the following text, cells grown in the presence of 50 ng/ml Dox that do not express hMLH1 and thus lack hMutLα will be referred to as 293T Lα$^-$ cells. Those grown in the absence of Dox, which express hMLH1 and thus contain functional hMutLα, will be referred to as 293T Lα$^+$ cells.

hMLH1 expression in 293T Lα cells restores MMR in vitro

Extracts of the 293T Lα cells were tested for MMR activity *in vitro* using two different MMR assays (see Materials and methods). No MMR activity was detected in extracts of 293T Lα$^-$ cells, but as the defect could be complemented by the addition of the recombinant wild-type hMutLα, we concluded that this heterodimer was the only factor missing in these extracts (Figure 2). In contrast, extracts from 293T Lα$^+$ cells were MMR proficient in both assays (Figure 2). Importantly, these results showed that the excess partnerless hMLH1 in the 293T Lα line does not inhibit MMR, at least not in our *in vitro* system. This differs from the situation in *Saccharomyces cerevisiae*, where overexpression of MLH1 gave rise to a mutator phenotype associated most likely with the inhibition of MMR through the homodimerization of this polypeptide (Shcherbakova and Kunkel, 1999; Shcherbakova *et al*., 2001). The MMR proficiency of the 293T Lα$^+$ cells in our *in vitro* assay was similar irrespective of whether the extracts were prepared from cells grown in the absence of Dox, or 24 h after the addition of the drug (data not shown), at which time point the ratio of hMLH1 to hPMS2 was 1:1 (Figure 1C).

Inducible hMLH1 expression restores sensitivity to alkylating agents

In order to determine the effect of hMLH1 expression on the sensitivity of 293T Lα cells to MNNG, we used clonogenic assays to quantify the surviving fraction of 293T Lα$^-$ and 293T Lα$^+$ cells following treatment with 5 μM MNNG. [Note that 293T Lα cells do not express MGMT, the enzyme responsible for the detoxification of methylation damage (G.Marra, unpublished data). For this reason, the experiments described below were carried out

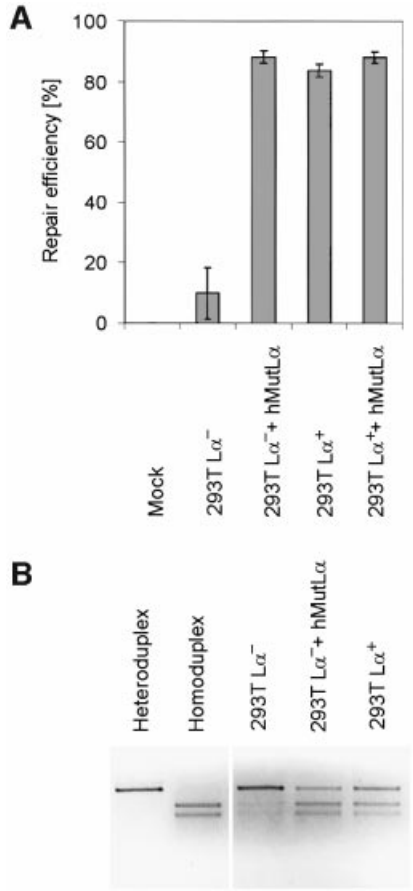
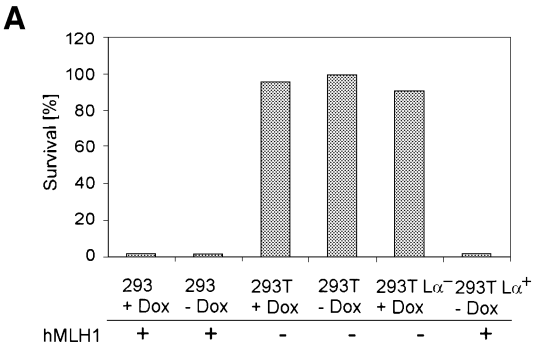


Fig. 2. MMR proficiency of 293T Lα cell extracts. (**A**) Repair efficiency of a G/T mismatch in the M13mp2 vector carrying a strand discrimination signal 3′ from the mispair, using cytoplasmic extracts of the 293T Lα$^+$ and 293T Lα$^-$ cells, supplemented or not with recombinant hMutLα (see text for details). Error bars show standard errors. (**B**) Correction of a G/T mismatch within a *Bgl*II restriction site of a pGEM vector, following incubation with nuclear extracts of 293T Lα$^+$ or 293T Lα$^-$ cells, supplemented or not with recombinant hMutLα. The strand discrimination signal in this heteroduplex substrate was 5′ from the mispair. Efficient repair resulted in the restoration of a *Bgl*II site and in the generation of two DNA fragments that co-migrate with those observed in the reference digest of the homoduplex molecule carrying a bona fide *Bgl*II site.

in the absence of the MGMT inhibitor O^6-benzylguanine.] As shown in Figure 3A, 293T Lα$^+$ cells were very sensitive to killing by MNNG, and the surviving fraction was indistinguishable from that obtained after MNNG treatment of the related MMR-proficient 293 cell line. In contrast, 293T Lα$^-$ cells were resistant to killing by MNNG, just like the parental, MMR-deficient 293T cells. The presence of Dox in the culture medium had no effect on the survival of any of the control cell lines used in this study (Figure 3A).

The sensitivity of 293T Lα cells to MNNG was further examined using the MTT assay, which is based on the cleavage of the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by the action of mitochondrial dehydrogenases to form a violet formazan dye. As this reaction takes place only in living cells, these can be distinguished from non-viable cells in a simple colorimetric assay. As shown in Figure 3B, 293T Lα$^-$ cells were 125-fold more resistant



B

	IC_{50} [μM MNNG]
293T Lα⁺	0.05 ± 0.003
293T Lα⁻	6.26 ± 0.500

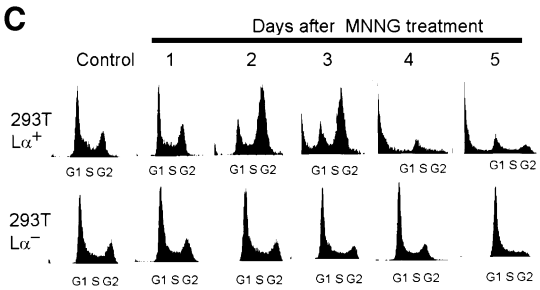


Fig. 3. Sensitivity of 293T Lα cells to MNNG. (**A**) Survival of 293T Lα$^+$ and 293T Lα$^-$ cells following treatment with 5 μM MNNG. 293 and 293T cells were used as MMR-proficient and -deficient controls, respectively. The presence of Dox (+Dox) in the culture medium did not affect the control cells, but had a dramatic effect on the survival of the 293T Lα cell populations. (**B**) IC_{50} values of 293T Lα$^+$ and 293T Lα$^-$ cells. Each value represents the mean ± SE. (**C**) Cell cycle profiles of 293T Lα$^+$ and 293T Lα$^-$ cells treated with 0.2 μM MNNG. Shown are representative cytometrograms of cells expressing (293T Lα$^+$) and not expressing (293T Lα$^-$) hMLH1. G_1, cell population in the G_1 phase of the cell cycle with a 2n DNA content; G_2, cells in the G_2 and M stages of the cell cycle with a 4n DNA content; S, cells in various stages of DNA synthesis with a DNA content between 2n and 4n.

to killing by MNNG than the same cells in a MMR-proficient mode (i.e. 293T Lα$^+$ cells).

Expression of hMLH1 in 293T Lα cells leads to activation of a methylation damage induced cell cycle arrest

To determine whether the increased sensitivity of 293T Lα$^+$ cells to MNNG resulted from induction of cell cycle arrest and cell death, the treated 293T Lα$^+$ and 293T Lα$^-$ cell populations were analysed by flow cytometry. As shown in Figure 3C, 2 days after treatment with 0.2 μM MNNG, the 293T Lα$^+$ cells were mostly arrested in the G_2/M phase of the cell cycle. One day later, cells containing sub-G_1 amounts of DNA became detectable, and this population increased with time. In contrast, no increase in the population of cells either arrested in G_2/M or with a lower than 2n DNA content was detected in cultures of treated 293T Lα$^-$ cells.

In order to further characterize the response of cells to MNNG, we analysed the phosphorylation status of Cdc2. As shown in Figure 4A, Cdc2 phosphorylated on Tyr15 accumulated exclusively in 293T Lα$^+$ cells treated with 0.2 μM MNNG. This provides molecular evidence for a G_2/M arrest, because so long as this kinase remains phosphorylated, entry into mitosis should be blocked. No difference in Cdc2 phosphorylation was observed in the extracts of MNNG-treated 293T Lα$^-$ cells (Figure 4A).

The above results thus show that induction of hMLH1 expression in the 293T Lα cells was necessary and sufficient to endow them with a MMR-proficient status, which also enabled them to respond to DNA damage induced by MNNG. What is presently unclear is the role of the MMR system in this checkpoint activation. DNA damage signalling is known to be mediated via several protein phosphorylation cascades, which involve primarily the DNA-dependent protein kinase (DNA-PK), or the ataxia telangiectasia-mutated (ATM) and ATM and Rad3-related (ATR) kinases. The downstream target of the latter enzymes is the p53 tumour suppressor protein, the phosphorylation of which on Ser15 is known to lead to its stabilization and subsequent activation as a transcription factor (Tibbetts *et al*., 1999). Phosphorylation of p53 has indeed been shown to take place upon MNNG treatment, and was shown to be dependent on functional hMutSα and hMutLα (Duckett *et al*., 1999; Hickman and Samson, 1999; Adamson *et al*., 2002). However, as the latter experiments were carried out with drug concentrations 25- to 125-fold higher than those used in our study, we wanted to test whether Ser15 phosphorylation also took place in the 293T Lα cells treated with 0.2 μM MNNG. These cells overexpress the SV40 large T antigen and thus contain large amounts of stabilized p53 polypeptide. This system is ideally suited for the study of post-translational modification of p53, as the steady-state levels of the latter protein remain unaltered during the experiment (Tibbetts *et al*., 1999). As anticipated, the p53 steady-state levels in the 293T Lα cell extracts were high, irrespective of whether hMLH1 was expressed or not, or whether extracts of treated or untreated cells were examined (Figure 4A). However, following MNNG treatment, phosphorylation of p53 with a Ser15-specific antibody could be detected exclusively in the MMR-proficient 293T Lα$^+$ cells. Notably, and in contrast to the study by Adamson *et al*. (2002), where the phosphorylation of p53 became detectable already just minutes after MNNG treatment, the MMR-dependent post-translational modification of p53 observed in our cells peaked at 48 h, i.e. at a time point where most cells were arrested at G_2/M (Figure 3C). This difference is probably linked with the high concentration of MNNG (25 μM) used in the latter study, which would be expected to introduce numerous single- and double-strand breaks into DNA that arise through the spontaneous loss of methylated purines and the subsequent breakage of the sugar-phosphate DNA backbone by β-elimination at the resulting abasic sites (Loeb, 1985). DNA strand breaks rapidly activate the ATM/ATR kinases that subsequently phosphorylate a number of downstream targets, one of which is histone H2AX (Redon *et al*., 2002). This histone modification is thought to aid the recruitment of DNA repair factors to the sites of damage (Paull *et al*., 2000). H2AX is phosphorylated in the 293T Lα cells upon

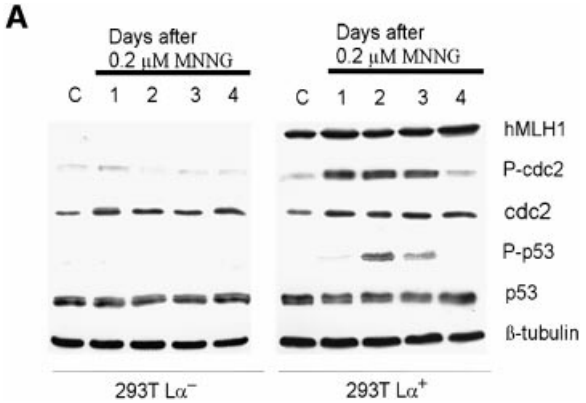
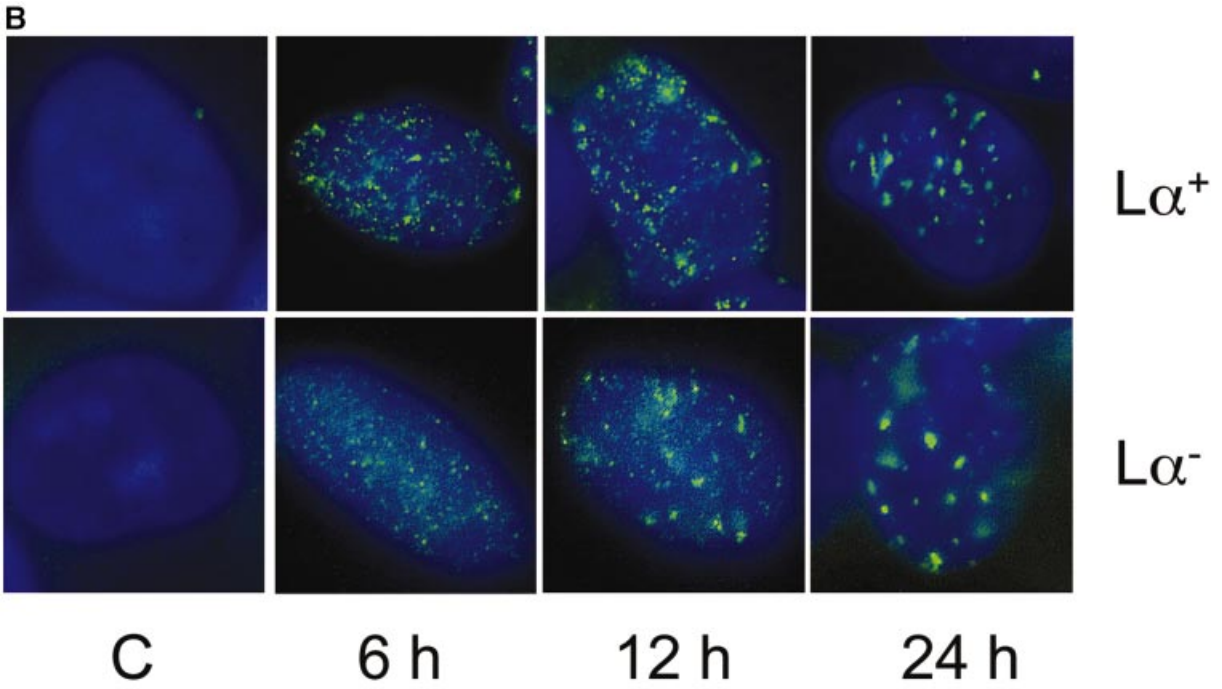


Fig. 4. Post-translational protein modification and strand break processing in MNNG-treated 293T Lα cells. (**A**) Phosphorylation status of p53 and cdc2 in 293T Lα$^+$ and 293T Lα$^-$ cells 1–4 days after treatment with 0.2 μM MNNG. P-p53, P-cdc2, phosphorylated p53 and cdc2 proteins, respectively; C, untreated control cells; β-tubulin, internal standard used to ascertain equal gel loading. (**B**) γ-H2AX foci formation in MNNG-treated 293T Lα cells. In the control cell population, <10% of cells displayed H2AX foci. Following MNNG treatment, all cells contained foci until 24 h post-treatment. See text for details and Materials and methods for experimental procedures.



treatment with 0.2 μM MNNG, as witnessed by the formation of phospho-H2AX foci (Figure 4B). However, these foci arise in both 293T Lα$^+$ and 293T Lα$^-$ cells soon after treatment. Thus, damage caused by direct modifications of DNA at low concentrations of MNNG does not trigger the G_2/M checkpoint. The activation of the checkpoint machinery must take place after H2AX phosphorylation, in the second cell cycle post-treatment (Kaina *et al*., 1997), and must involve the MMR system, perhaps in conjunction with another pathway of DNA metabolism that remains to be identified. Thus, the lesions that trigger the checkpoint machinery are distinct from those that bring about phosphorylation of H2AX.

MMR proficiency and response to MNNG treatment require different levels of hMLH1 expression

The principal goal of this study was to investigate the phenotypic effects of reduced expression of MMR proteins, such as might be encountered when expression of the gene is attenuated by cytosine methylation. In order to achieve this goal, we attempted to modulate hMLH1 expression in the 293T Lα cells. This could be achieved by varying the Dox concentration in the culture media. Thus, cells grown in the presence of 0.1, 0.2, 0.4, 0.8 and 1.5 ng/ml Dox contained steadily decreasing amounts of hMLH1 and hPMS2, as compared with cells grown in the absence of the drug (Figure 5A).

When we tested how this variation in the amount of hMutLα affected MMR efficiency, we found that extracts of cells expressing as little as 10% of the amounts found in cells grown in the absence of Dox were still proficient in the *in vitro* MMR assays. Cells cultivated with 0.1 and 0.2 ng/ml Dox showed MMR activities comparable to those of the MMR-positive 293T Lα$^+$ cells grown in the absence of Dox, and even extracts of cells cultivated with 0.4 ng/ml Dox were still able to repair mismatches *in vitro*, albeit with lower efficiency (Figure 5B). MMR proficiency was lost only in cell extracts in which the hMLH1 and hPMS2 proteins became difficult to detect by western blotting (Figure 5A). To test whether the results of the *in vitro* MMR assays were reflected also in the MSI

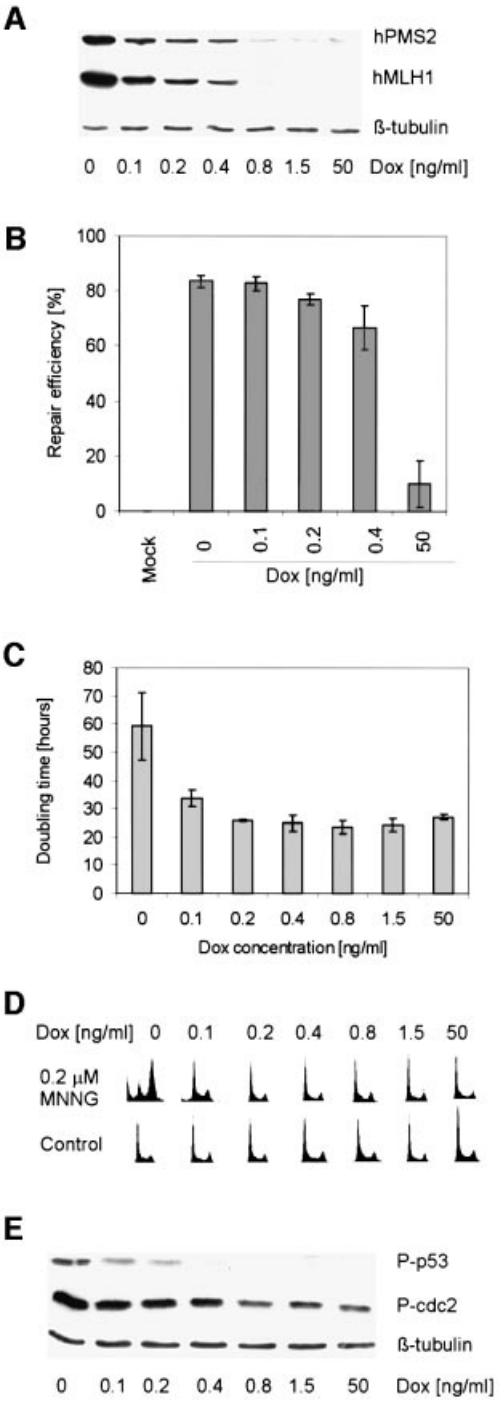


Fig. 5. Mismatch correction efficiency and MNNG-induced G_2/M arrest in cells expressing different amounts of hMLH1. (**A**) Dependence of hMLH1 expression on Dox concentration. hMLH1 and hPMS2 were visualized as described in Materials and methods. β-tubulin, internal standard used to ascertain equal loading. (**B**) MMR efficiency of a G/T mispair in an M13mp2 substrate carrying a strand-discrimination signal 3′ from the mispair. Error bars show standard errors. (**C**) Variation in doubling times of 293T Lα cells grown in the indicated Dox concentrations following treatment with 5 µM MNNG. (**D**) FACS analysis of 293T Lα cell populations grown in the indicated Dox concentrations, either untreated (Control), or 72 h after treatment with 0.2 µM MNNG (see also Figure 3C). (**E**) Phosphorylation of p53 and cdc2 48 h after treatment of cells (grown in the indicated Dox concentrations) with 0.2 µM MNNG. β-tubulin, internal standard used to ascertain equal loading.

Table I. Instability of the BAT26 chromosomal locus in 293T Lα cells expressing varying amounts of hMLH1

Dox (ng/ml)	MSI+/total	% MSI
0	2 (0)/131	1.5
0.2	1 (0)/80	1.3
50	4 (2)/73	5.5 (2.7)

MSI+ clones were defined as those displaying more than three extra peaks in the sequence of the PCR product. Numbers in parentheses refer to clones with more than four extra peaks.

phenotype of the cells, we analysed the BAT26 microsatellite marker, which contains a repeat of 26 deoxyadenosines, and which is considered to be a reliable indicator of MSI. Because the 293T Lα cells are hypotriploid, and because this cell line was MMR deficient for many generations prior to our intervention, the BAT26 locus was found to be highly heterogeneous. The product of PCR amplification had on average eight peaks, and we therefore applied the HNPCC criteria of MSI (Loukola *et al.*, 2001), whereby only PCR products that differed by three or more peaks at this locus were considered to be a sign of MSI. By these criteria, the BAT26 instability in the cells propagated for 35 generations in 0 or 0.2 ng/ml Dox was ~1%, whereas cells grown with 50 ng/ml Dox displayed MSI that was ~5-fold higher (Table I). However, closer inspection of the data revealed that cells propagated in 0 or 0.2 ng/ml Dox displayed no alleles (0/211) that differed by more than 4 bp from the median. In contrast, two such alleles (two out of 73; 2.7%) were found in the cells grown with 50 ng/ml Dox (Table I, numbers in parentheses). This suggests that MSI at the BAT26 locus in the 293T Lα⁻ cells is substantially greater than in cells expressing hMLH1, and thus that expression of even low amounts of hMutLα are sufficient to correct the MMR defect in these cells, both *in vitro* (Figure 2) and *in vivo* (Table I).

We were interested to determine whether the low amounts of the hMLH1/hPMS2 heterodimer that were shown to restore MMR proficiency were also able to activate the DNA damage-induced cell cycle arrest in 293T Lα cells. To this end, we treated the cells with 5 µM MNNG and calculated the average doubling time over a period of 5 days. In accordance with our previous experiments, only cells expressing the highest amounts of hMLH1 (i.e. 293T Lα+ cells grown without Dox) ceased to grow, as suggested by their increased doubling time. Cells grown in 0.1 ng/ml Dox were only partially affected, and cells cultivated with 0.2 ng/ml Dox or more grew similarly to 293T Lα⁻ cells (Figure 5C). To test whether this growth retardation was due to checkpoint activation, we analysed the DNA content of the cells 3 days after treatment with 0.2 µM MNNG. As shown in Figure 5D, FACS analysis showed that only cells expressing the highest amounts of hMLH1 (i.e. cells cultured without Dox) displayed a strong G_2/M arrest (an average of 63% of the cells were in G_2/M). The response of cells cultivated with 0.1 ng/ml Dox was substantially weaker (~27% cells in G_2/M), and the cell cycle profiles of cells grown with 0.2 ng/ml Dox or more were indistinguishable from those of the untreated controls (~22% cells in G_2/M). Notably, whereas cells grown in the absence of Dox activated the MNNG-induced G_2/M checkpoint, while those grown in

0.2 ng/ml Dox failed to do so, phosphorylated forms of p53 and cdc2 could be detected in both cell populations (Figure 5E). The extent of cdc2 phosphorylation in particular would predict that a detectable proportion of the treated cells should be at the G_2/M boundary. This was clearly not the case, as judged by FACS analysis (Figure 5D; also see Figure 3C).

Taken together, these experiments show that although only low amounts of hMutLα are required for MMR proficiency, DNA damage-induced G_2/M arrest and cell death in response to MNNG treatment require a full complement of this heterodimer. The fact that the 293T Lα$^+$ cells arrest and die with kinetics and efficiency similar to other MMR-proficient cells confirms that p53, which is inactive in these cells, is not required for either of these processes (Hickman and Samson, 1999). Thus, the molecular pathways controlling the MNNG-induced G_2/M checkpoint in these cells require further study.

Discussion

We show that expression of hMLH1 in 293T Lα cells corrected their MMR defect *in vitro* and *in vivo*. The 293T Lα$^+$ cells were also found to be >100-fold more sensitive to killing by MNNG than the isogenic cells lacking hMLH1. MNNG treatment arrested the MMR-proficient cells in the G_2/M phase of the cell cycle, and this arrest was entirely and solely dependent on the function of hMLH1. This latter statement is supported by the finding that expression of hMLH1 in 293T Lα$^+$ cells did not affect the transcriptional activity of other genes, as demonstrated by Affymetrix GeneChip™ analysis (data not shown).

This study also showed that the steady-state levels of the hMLH1/hPMS2 heterodimer required for MMR proficiency and DNA damage response were significantly different. In earlier experiments (Lettieri *et al*., 1999) we generated a cell line derived from hMSH6-deficient HCT15 cells, which expressed low levels (~20%) of wild-type hMSH6. This line was MMR proficient, but remained as resistant to killing by methylating agents as the parental cell line. Similarly, a recent study described a *Msh2*$^{-/-}$ mouse embryonic stem cell line in which the MMR defect was largely corrected by the expression of low levels (10% of control) of exogenous Msh2, but the response of these cells to methylating agents was comparable to that observed with the parental *Msh2*$^{-/-}$ cells (Claij and Te Riele, 2002). This damage signalling defect was suggested by the authors to be linked to poor recognition of ^{Me}G/T mispairs, which arise through the mispairing of O^6-methylguanine (^{Me}G) with thymine during DNA replication (Karran and Bignami, 1996), and which are bound less efficiently than bona fide mispairs by the hMSH2/hMSH6 (hMutSα) heterodimer (Duckett *et al*., 1996). Constant loading of hMutSα sliding clamps at ^{Me}G/T mispairs was proposed be responsible for transmission of the DNA damage signal to the checkpoint machinery *in vivo* (Fishel, 1999), and it might be expected that this process is substantially less efficient in cells expressing only low amounts of the mismatch binding factor hMutSα. However, the amounts of hMutSα in 293T Lα$^+$ and 293T Lα$^-$ cells are equal, and similar to those found in other MMR-proficient cells. Our results thus extend the above hypothesis by showing that the signal transduction process also requires the hMLH1/hPMS2 heterodimer, thought to act downstream of damage recognition. Moreover, our result show that the recognition of ^{Me}G/T mispairs *per se* is insufficient to activate the checkpoint machinery. The G_2/M checkpoint is thought to be controlled by the phosphoinositide-3 (PI3) kinases ATM/ATR, which are principally responsible for the phosphorylation of p53 on Ser15 (Osborn *et al*., 2002). The ^{Me}G/T mispairs arise already during the first round of replication, yet no p53 phosphorylation is detectable until 24 h after treatment, at which point the cells are beginning to enter the second S phase (Figure 3C; data not shown). Notably, the peak of signalling activity coincides with that of chromosomal rearrangements (sister chromatid exchanges and recombinations) induced by MNNG (Kaina *et al*., 1997). Thus, MMR-dependent processing of the ^{Me}G/T mispairs that arise during the first S phase apparently does not activate the checkpoint machinery, but leads instead to the generation of intermediates that result in aberrant recombination events during the subsequent round of DNA replication, which then signal. What the exact nature of these intermediates may be is currently the subject of intensive studies.

The evidence presented here shows that cells with lower than wild-type levels of MMR proteins are not phenotypically normal, despite being MMR proficient. The observed defect in DNA damage signalling may be relevant to cellular transformation and cancer, particularly in epithelial cells that are rapidly proliferating and that may be exposed to stress or carcinogens. In the colon, the epithelial stem cells that are near the bottom of the crypts give rise to daughter cells that begin to differentiate during their migration towards the surface of the colon. Upon reaching the apex of the crypt, these cells undergo apoptosis and are shed. When the colonic epithelial cells become damaged, they should undergo apoptosis and thus give rise to no mutant progeny. In contrast, cells with a defect in DNA damage signalling, such as those expressing suboptimal amounts of MMR proteins, would not activate cell cycle checkpoints and apoptosis in response to DNA damage. Instead, they might acquire mutations that allow them to continue to proliferate and give rise to an adenoma.

The relevance of this hypothesis to the situation *in vivo* hinges on two points. First, there are currently no experimental data documenting instances where colonocytes or other epithelial cells that are prone to transformation express low MMR protein levels. We obtained some evidence of lower than normal steady-state levels of hMSH2 and increased resistance to methylating agents in the immortalized lymphoblasts of HNPCC patients, which are heterozygous in the *hMSH2* locus, but the *hMLH1*$^{-/-}$ cells were normal in all assays (Marra *et al*., 2001). It is not known whether hMSH2 and hMLH1 levels in heterozygous colonocytes of HNPCC kindred are lower than in similar cells of normal individuals, even though some fluctuations might be expected. However, the recent characterization of the *hMLH1* promoter as a frequent target of DNA hypermethylation (Esteller, 2002) implies that there must be cells with only partially methylated promoters, because *de novo* methylation of CpG islands is a gradual process. These cells, such as the 293T Lα cells grown in low concentrations of Dox (Figure 5A), would

contain decreased levels of hMutLα and would therefore be likely to also have a defective response to DNA damage.

The second point concerns the nature of the endogenous DNA damage that might trigger the transformation process. It is conceivable that normal colonocytes which become damaged by endogenous or exogenous DNA modifying agents would arrest and, in cases where the extent of the damage is beyond repair, activate cell death processes, while those expressing reduced levels of hMutLα would continue to proliferate and thus acquire mutations. Although human DNA is aberrantly modified by *S*-adenosyl methionine and other methyl group donors, the extent of such modifications might be too low to trigger cell death. However, the deleterious effects of the checkpoint defect could become evident also in response to other types of DNA damage; experimental evidence implicates the MMR system in the processing of DNA modifications ranging from oxidative damage to bulky moieties such as cisplatin and AAF (reviewed in Bellacosa, 2001).

We have described a cell line in which the MMR status can be controlled by the concentration of doxycycline in the culture medium. Our current results show that the activation of transcription of exogenous *hMLH1* complements not only the MMR defect of the 293T cells, but also reactivates their responsiveness to treatment with methylating agents, providing that the levels of the MMR proteins are sufficiently high to activate the DNA damage-induced checkpoint. This fully isogenic system is clearly open to further exploitation, and should allow us to study the involvement of the MMR system in other pathways of DNA metabolism, such as response to other types of DNA damaging agents ranging from ionizing radiation to crosslinking chemotherapeutics, where the involvement of MMR was found to be only marginal and where it could not be ruled out that the observed effects (or lack thereof) were linked to a selection of an atypical clone from the stably transfected population. The 293T Lα line could also be used in the screening for substances that preferentially kill MMR-deficient cells. This should prove invaluable in the treatment of tumours, both hereditary and sporadic, with defective MMR.

Materials and methods

Cell lines

The 293T cells (a kind gift of K.Ballmer) were grown in Dulbecco's modified Eagle's medium with Eagle salts (Gibco-BRL, Gaithersburg, MD), supplemented with 10% Tet System Approved Fetal Bovine Serum (Clontech, Palo Alto, CA), 2 mM L-glutamine (Gibco-BRL), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco-BRL). For 293T-TetOff or 293T Lα cells, 100 µg/ml Zeocin (Invitrogen, San Diego, CA) or 100 µg/ml Zeocin and 300 µg/ml Hygromycin B (Roche Molecular Biochemicals, Mannheim, Germany) were added, respectively.

Plasmid construction

The pTetOff-Zeo plasmid was constructed by ligation of the following DNA molecules: the first, coding for tTA, was obtained by digestion of pTetOff (Clontech) with *Xho*I (New England Biolabs, Beverly, MA) followed by filling-in with dCTP and dTTP using the Klenow fragment of DNA polymerase I (New England Biolabs). The second, coding for Zeocin resistance protein, was obtained by digestion of pVgRXR (Invitrogen) with *Bam*HI (New England Biolabs) followed by filling-in with dGTP and dATP. The pTRE2-hMLH1 plasmid was generated by subcloning hMLH1 cDNA (a kind gift of R.Michael Liskay) into the *Bam*HI and *Not*I sites of pTRE2 (Clontech).

Calcium phosphate transfections

One day before transfection, 250 000 cells were plated in 6-well plates in 3 ml of cell culture medium. The cells reached ~50% confluency on the day of transfection. Three hundred microlitres of solution A (250 mM $CaCl_2$) was carefully mixed with 15 µg DNA and 300 µl of solution B (140 mM NaCl, 50 mM HEPES, 1.4 mM Na_2HPO_4 pH 7) in an Eppendorf tube. Exactly 1 min after mixing, 300 µl of the precipitation cocktail was added to the medium. The plates were incubated for 4 h at 37°C. The medium was then removed, the cells were washed with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM KH_2PO_4) and fresh cell culture medium was added.

Generation of the 293T Lα cell line

293T cells were transfected with pTetOff-Zeo using the calcium phosphate method (see above). The selection of stable cell lines was initiated 2 days later using 400 µg/ml Zeocin. After 3 weeks, ~50 colonies were isolated and screened by transient transfection with pTRE2-Luc (Clontech) for the expression of luciferase in induced and noninduced cells (with or without 2 µg/ml Dox; Clontech). The clone with the lowest background and high induction of luciferase (293T-TetOff) was then transfected with pTRE2-hMLH1 and pTK-Hyg (ratio 15:1). Selection of stable cell lines was initiated 2 days post-transfection using 400 µg/ml hygromycin-B. After 3 weeks, ~160 colonies were isolated and their extracts were screened by western blotting using antibodies against hMLH1, hPMS2 and β-tubulin. The clone 293T Lα was selected for further study, as it displayed the highest induction of hMLH1 in the absence of Dox, and no background expression with 2 µg/ml Dox.

Regulation of hMLH1 expression

293T Lα cells were grown in the presence of 50 ng/ml Dox to keep hMLH1 expression turned off; fresh Dox was added every second day. To induce hMLH1 expression, the cells were transferred to a Dox-free medium, and the cells were cultivated for at least 6 more days. To obtain cells completely free of hMLH1, cells grown in the absence of Dox were kept for a least 7 days in a medium containing 50 ng/ml Dox. To obtain intermediate levels of hMLH1, the cells were cultivated with 1.5, 0.8, 0.4, 0.2 or 0.1 ng/ml Dox for at least 7 days.

Preparation of total protein extracts for western blots

Cells were harvested, transferred to a 1.5 ml Eppendorf tube and washed twice with PBS. Cell lysis was performed on ice in 50 mM Tris–HCl pH 8, 125 mM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1× complete protease inhibitory cocktail (Roche Molecular Biochemicals) for 25 min. Insoluble material was pelleted by centrifugation at 18 000 *g* for 3 min at 2°C. Protein concentration was determined using the Bradford assay (Bio-Rad, Munich, Germany).

Western blot analyses

The primary antibodies used in this study were: anti-hMLH1 [PharMingen, San Diego, CA), 1:2000 in TBST (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20 with 2.5% non-fat dry milk], hPMS2 (Calbiochem; 1:500), β-tubulin, p53 (Santa Cruz Biotechnology; 1:1500 and 1:2000, respectively), cdc2 (Upstate Biotechnology; 1:1000) and phospho-p53-Ser15, phospho-cdc2-Tyr15 (Cell Signalling Technology; 1:1000 and 1:5000, respectively). The proteins (20–50 µg) were denatured, reduced, separated by SDS–PAGE (7.5–12.5%) and transferred to Hybond-P PVDF membrane (Amersham Pharmacia Biotech) according to standard protocols (Sambrook *et al*., 1989). The membranes were blocked with 5% non-fat dry milk in TBST for 60 min, incubated with primary antibodies for 60 min, washed three times with TBST for 10 min, incubated with the peroxidase-conjugated secondary antibody (anti-mouse IgG, 1:5000 in TBST with 2.5% non-fat dry milk) for 60 min and washed three times with TBST for 10 min. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

Indirect immunofluorescence experiments

Cells grown on coverslips were treated or mock-treated with MNNG (0.2 µM end concentration) and incubated for 6, 12 and 24 h (Figure 4B). Foci of phosphorylated histone H2AX were visualized using an anti-phospho-H2AX rabbit polyclonal antibody (Upstate Biotechnology) at +4°C, over night, at a dilution of 1:100. The procedure was as described previously (Kleczkowska *et al*., 2001). To allow direct comparisons, all

the cells were treated and processed simultaneously, and all the images were obtained using the same magnification, brightness and contrast settings.

MMR assays

The cell extracts were prepared as described previously (Marra *et al*., 2001; Nystrom-Lahti *et al*., 2002). Two different *in vitro* assays were used. The first, adapted from Holmes *et al*. (1990), is based on a circular 3′ 193 bp DNA molecule containing a G/T mismatch within a unique *Bgl*II recognition site, a single-strand nick 369 nucleotide residues 5′ from the mismatch in the G-containing strand, and a unique *Bsa*I site. This molecule is refractory to cleavage with *Bgl*II, unless the mispair is corrected to an A/T. Thus, the unrepaired heteroduplex digested with both endonucleases gives rise to only a single fragment of 3′ 193 bp, whereas the repaired homoduplex is cleaved into two fragments of 1′ 833 and 1′ 360 bp (Nystrom-Lahti *et al*., 2002).

The second method, originally described by Thomas *et al*. (1991), makes use of an M13mp2 DNA heteroduplex containing G/T mismatch within lacZα complementation gene, obtained by hybridizing single-stranded viral (+) DNA with the replicative form I (–) strand. The repair is directed to the (–) strand by the presence of a nick. The method was described in detail elsewhere (Marra *et al*., 2001). In the complementation studies, extracts were supplemented with purified recombinant hMutLα (0.1 μg).

MTT assays

Two thousand cells/well were plated in 96-well plates, treated the next day with various concentrations of MNNG (Sigma; diluted in dimethyl sulfoxide and stored at –20°C in the dark) and incubated for 5 days. Then, 20 μl of MTT solution (5 mg/ml MTT; Sigma; in PBS, sterile filtered) was added, and the plates were incubated for 4–5 h at 37°C. One volume of lysis solution was then added (20% SDS, 50% dimethylformamide pH <4.7), and the plates were incubated overnight at 37°C. The solubilized formazan was quantified at 570 nm, using the Versamax microplate reader (Molecular Devices, Sunnyvale, CA). The optical density values were plotted against logarithm of MNNG concentrations and IC_{50} values were calculated from the regression curve.

Colony-forming assays

Cells in log phase (50–80% confluent) were treated with 5 μM MNNG, harvested after 2 h, and 200 or 2000 cells per duplicate were plated in 10 cm plates. Colonies were counted after 15–20 days of incubation. Survival was calculated as the ratio of the number of colonies from treated versus untreated samples.

Doubling time assessment

Cells (35 000) were plated in 35 mm plates. The cell number was determined daily for 4 days. The doubling time was calculated from the numbers of cells between the first and the fourth day after plating.

Cell cycle analyses

Cells (both attached and floating) were harvested, counted, washed with PBS, fixed with 70% ethanol and stored up to 1 week at 4°C. The cells were then washed once with PBS, incubated in PBS containing RNase A (100 μg/ml, Sigma) for 1 h at 37°C, stained with propidium iodide (20 μg/ml, Sigma) and incubated on ice in the dark for 30 min. DNA content was analysed by Coulter Epics Altra Flow Cytometer (Beckman Coulter, Inc., Fullerton, CA). DNA cell cycle analysis software (MultiCycle, Phoenix Flow Systems, Inc., San Diego, CA) was used to quantify cell cycle distribution.

MSI analysis

293 Lα cells grown with 50, 0.2 and 0 ng/ml Dox were subcloned, and grown independently for 35 generations. The chromosomal DNA was extracted using the TRI Reagent (Molecular Research Center, Lucerne, Switzerland). MSI was assessed at the mononucleotide repeat locus BAT26. PCRs were carried out in a total volume of 25 μl containing ~100 ng of genomic DNA, as described by Loukola *et al*. (2001). The PCR products were diluted 1:4 and 0.5 μl was added to 10 μl deionized formamide (including 0.5 μl GS size standard 400 ROX), denatured at 95°C for 5 min, chilled on ice and loaded on a 96-capillary ABI PRISM 3700 DNA Analyzer (PE Applied Biosystems). MSI was defined as the occurrence of novel alleles that differed by ±3 nucleotides from the control (Loukola *et al*., 2001).

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4.2 Mismatch repair-dependent G2 checkpoint induced by low doses of S_N1 type methylating agents requires the ATR kinase

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Functional absence of MMR proteins has been linked to defective DNA damaging signalling and cell cycle arrest upon MNNG treatment. In order to study the role of MMR proteins in activation of signalling cascades, 293T Lα cells were treated with MNNG and subsequent activation of DNA damage signalling pathways was assessed. Stojic *et al.* show that MNNG treatment induced a cell cycle arrest that was absolutely dependent on functional MMR. Unusually, the cells arrested only in the second G_2 phase after treatment. Downstream targets of both ATM and ATR kinases were modified, but only the ablation of ATR, or the inhibition of CHK1, attenuated the arrest. The checkpoint activation was accompanied by the formation of nuclear foci containing the signaling and repair proteins ATR, the S*/T*Q substrates, γ-H2AX, and replication protein A (RPA). The persistence of these foci implied that they might represent sites of irreparable damage.

Mismatch repair-dependent G_2 checkpoint induced by low doses of S_N1 type methylating agents requires the ATR kinase

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S_N1-type alkylating agents represent an important class of chemotherapeutics, but the molecular mechanisms underlying their cytotoxicity are unknown. Thus, although these substances modify predominantly purine nitrogen atoms, their toxicity appears to result from the processing of O^6-methylguanine (^{6Me}G)-containing mispairs by the mismatch repair (MMR) system, because cells with defective MMR are highly resistant to killing by these agents. In an attempt to understand the role of the MMR system in the molecular transactions underlying the toxicity of alkylating agents, we studied the response of human MMR-proficient and MMR-deficient cells to low concentrations of the prototypic methylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). We now show that MNNG treatment induced a cell cycle arrest that was absolutely dependent on functional MMR. Unusually, the cells arrested only in the second G_2 phase after treatment. Downstream targets of both ATM (*Ataxia telangiectasia* mutated) and ATR (ATM and Rad3-related) kinases were modified, but only the ablation of ATR, or the inhibition of CHK1, attenuated the arrest. The checkpoint activation was accompanied by the formation of nuclear foci containing the signaling and repair proteins ATR, the S*/T*Q substrate, γ-H2AX, and replication protein A (RPA). The persistence of these foci implied that they may represent sites of irreparable damage.

[*Keywords*: ATM/ATR; cell cycle arrest; DNA damage signaling; G_2 check point; methylating agents; mismatch repair]

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Treatment of cells with clastogenic DNA damaging agents such as ionizing radiation (IR) generally results in the rapid activation of damage signaling pathways, cell cycle arrest and, depending on the extent of damage, either recovery or cell death. IR causes predominantly DNA base modifications (Cooke et al. 2003), which are rapidly and efficiently processed by the base excision repair (BER) system. Interestingly, this metabolic pathway does not appear to trigger DNA damage checkpoints. Instead, IR-induced signaling events are believed to be associated exclusively with the detection or processing of single- and double-strand breaks (DSBs), which rapidly activate the ATM (*Ataxia telangiectasia* mutated) kinase (Bakkenist and Kastan 2003) and, later, also ATR (ATM and Rad3-related; Brown and Baltimore 2003). DNA damage-induced signaling cascades can be activated also by DNA replication forks stalled by DNA damage (e.g., ultraviolet-induced photodimers or cross-links), nucleotide depletion (e.g., on hydroxyurea treatment), or polymerase arrest (e.g., by aphidicolin). In all the latter cases, the signaling events are triggered in the first S phase after treatment and involve primarily the activation of ATR kinase and its downstream targets (Abraham 2001; Osborn et al. 2002; Shiloh 2003).

DNA damage signaling induced by S_N1-type methylating agents has to date not been studied in detail. Treatment of cells with *N*-methyl-*N*-nitrosourea (MNU) and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) gives rise predominantly to N^7-methylguanine (^{7Me}G), N^3-methyladenine (^{3Me}A), O^4-methylthymine (^{4Me}T), O^6-methylguanine (^{6Me}G), and methyl-phosphotriesters in their DNA. The major adducts, ^{7Me}G and ^{3Me}A, represent ~70% of the damage. However, both these methylated bases are efficiently removed from DNA by alkyladenine DNA-glycosylase (Scharer and Jiricny 2001), and the resulting abasic sites are repaired by the BER pathway (Seeberg et al. 1995), without causing undue cytotoxicity at low concentrations. Interestingly, the cytotoxicity of the above methylating agents is ascribed to ^{6Me}G, detoxified by methylguanine methyl transferase (MGMT), which reverts it back to guanine (Sedgwick

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and Lindahl 2002). ^{6Me}G residues were implicated in cell killing when cells expressing high levels of MGMT were shown to be highly resistant to killing by MNU (Karran 2001), but how can persistent ^{6Me}G residues in DNA lead to cell death?

In 1993, the presence of ^{6Me}G in plasmid DNA was shown to inhibit DNA replication, but also to stimulate DNA repair synthesis (Ceccotti et al. 1993). This evidence was extended to show that ^{6Me}G residues did not inhibit DNA polymerases per se, but that DNA replication was arrested through a *trans*-acting signal generated during the processing of ^{6Me}G residues in DNA (Zhukovskaya et al. 1994). The discovery that cells defective in both mismatch repair (MMR) and MGMT were resistant to killing by methylating agents implicated the MMR system in this processing. The MMR substrates are thought to be ^{6Me}G/T mispairs, which arise during replication of methylated DNA because of the propensity of ^{6Me}G to preferentially base pair with thymine. The recognition of the ^{6Me}G/T mispairs by the mismatch binding factor hMutSα (Duckett et al. 1996) is believed to activate a signal transduction pathway that results in a G_2/M arrest (Aquilina et al. 1999; Cejka et al. 2003). However, how this arrest is activated is currently unclear. One hypothesis proposes that the repeated loading of the mismatch binding proteins at the mismatch site may be sufficient to activate a DNA damage-signaling cascade (Fishel 1998). The other suggests that the cell cycle arrest is activated by nonproductive, repetitive processing of ^{6Me}G/T mispairs by the MMR system, or by intermediates arising as a result of this processing (for review, see Bellacosa 2001; Karran 2001). We set out to gain more insights into the molecular transactions underlying the G_2/M cell cycle arrest induced by methylating agents of S_N1 type. To this end, we studied the behavior of proteins involved in DNA damage signaling and processing in a cell line in which MMR status can be tightly controlled (Cejka et al. 2003).

Results

MNNG-induced MMR-dependent G_2 arrest occurs in the second cell cycle

The human embryonic kidney cell line 293T is MMR-deficient and does not convert ^{Me}G in its DNA back to G, as the promoters of the *hMLH1* (Trojan et al. 2002) and *MGMT* (Cejka et al. 2003) genes are epigenetically silenced. We used these cells to generate the 293T Lα cell line, which carries a stably integrated *hMLH1* cDNA minigene controlled by the TetOff expression system. In the absence of doxycycline (Dox), these cells, referred to as 293T Lα$^+$, express hMLH1, are MMR-proficient, and are sensitive to killing by MNNG (Cejka et al. 2003; Di Pietro et al. 2003). In contrast, when the same cells are grown in the presence of 50 ng/mL Dox (293T Lα$^-$ cells), they shut off hMLH1 expression, display a MMR defect, and are 125-fold more resistant to MNNG than 293T Lα$^+$ cells. Flow cytometric analysis showed that on treatment with 0.2 μM MNNG, the 293T Lα$^+$ cells arrested with a DNA content of 4n (Cejka et al. 2003). Interestingly, the arrest did not take place in the first cell cycle, as synchronized 293T Lα cells treated with MNNG at the G_1/S-transition progressed through the first G_2/M boundary and mitosis irrespective of their MMR status. The arrest was activated after the second S phase, and only in the MMR-proficient 293T Lα$^+$ cells (Fig. 1A). However, 293T cells express the SV40 large T antigen, as well as the adenoviral E1A and E1B proteins, which inhibit the functions of the retinoblastoma (Rb) and p53 tumor suppressor proteins in regulating the G_1/S transition on DNA damage (Bartek and Lukas 2001). To ensure that the proper functioning of DNA damage response in 293T Lα$^+$ cells was not affected, and to show that the observed arrest in the second cell cycle was not limited to this cell line, we repeated this experiment with synchronized HCT116 (hMLH1-deficient) and HCT116 + ch3 (hMLH1-proficient) cells that carry both functional p53 and pRb. As shown in Figure 1B, both these latter cell lines proceeded through the first cell cycle in a similar manner. However, 20 h posttreatment, the MMR-proficient HCT116 + chr3 cells began to accumulate in the second S phase and then proceeded to arrest with a DNA content of 4n (T30), whereas the MMR-deficient HCT116 cells exited the second S phase normally and continued to cycle.

We next had to confirm that the cells indeed arrested in the G_2 phase of the cell cycle, rather than stopping because of a mitotic catastrophe. To this end, we added nocodazole, an inhibitor of mitotic spindle formation, to the MNNG-treated cell cultures 24 h before cytological analysis. If the treated cells were arrested in G_2, they could not traverse to mitosis. Thus, nocodazole should block only cells that failed to arrest and continued to cycle. As shown in Figure 1C, the MMR-deficient 293T Lα$^-$ cells treated first with MNNG and then with nocodazole were frequently arrested in mitosis. This indicates that they did not arrest before this phase. In contrast, when nocodazole was added to the MNNG-treated MMR-proficient 293T Lα$^+$ cells, the number of cells reaching mitosis was substantially lower, which shows that more MMR-proficient cells preferentially arrested in G_2 after MNNG treatment. The finding that arrest took place after the second S phase was further confirmed by bromodeoxyuridine (BrdU) labeling experiments, in which synchronized, MNNG-treated 293T Lα cells were shown to enter the second S phase between 14 and 24 h after treatment, irrespective of their MMR status (Fig. 1D). As shown in the graph, the number of cells in the second S phase appeared lower than in the first. To see whether some cells died during the course of this experiment and were therefore lost, we followed the proliferation of the unsynchronized, MNNG-treated cell populations. As shown in Figure 1E, no appreciable cell loss occurred: The MNNG-treated MMR-proficient cells doubled in number during the first 24 h and then arrested, whereas the treated MMR-deficient cells continued to proliferate. This showed that the decrease in cell number in the second S phase (Fig. 1D) was only apparent and was most likely the result of the gradual loss

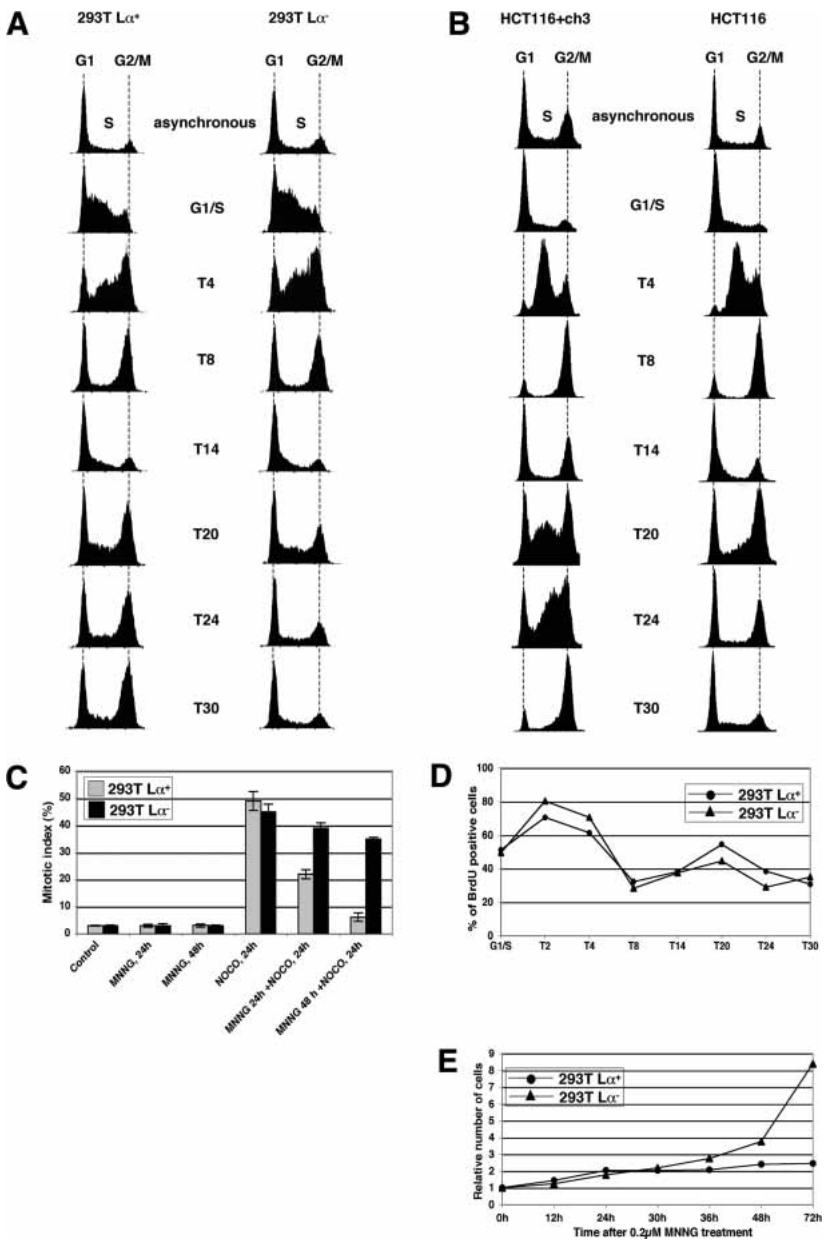

Figure 1. Kinetics of the G_2/M arrest in cells treated with 0.2 μM MNNG. (*A*) FACS analysis of 293T Lα cell cultures synchronized in G_1/S with a double thymidine block and treated with 0.2 μM MNNG. (T4–T30) FACS analyses carried out 4–30 h posttreatment. (*B*) FACS analysis of MNNG-treated HCT116 and HCT116 + ch3 cell cultures synchronized in G_1/S with HU. (T4–T30) FACS analyses carried out 4–30 h posttreatment. The 4n peak in the unsynchronized and HU-synchronized HCT116 + chr3 cells is larger than in the HCT116 cells. This is not the result of a larger fraction of diploid cells in the G_2 phase of the cell cycle, but to a subpopulation of tetraploid cells, which arrest after MNNG treatment with a content of 8n (not shown). (*C*) Mitotic index of 293T Lα cells after MNNG treatment. The cells were treated with MNNG, and nocodazole was added 24 or 48 h later. The cells were microscopically examined after an additional 24 h. As shown, substantially more MMR-deficient 293T Lα$^-$ cells were arrested in mitosis than were MMR-proficient 293T Lα$^+$ cells, which indicates that the latter cells were more frequently arrested in G_2. (*D*) Synchronized 293T Lα cells (as in *A*) were pulse-labeled with BrdU, and the number of cells in S phase was estimated by CELLQuest software. Both 293T Lα$^+$ (●) and 293T Lα$^-$ (▲) cells entered the second S phase between T14 and T24. (*E*) Growth curves of unsynchronized MNNG-treated 293T Lα cells. Although the treated 293T Lα$^+$ cells doubled their number 24 h after treatment and then ceased to proliferate, the 293T Lα$^-$ cells continued to grow.

of synchronization. In summary, the MNNG-induced checkpoint in MMR-proficient cells is activated after the second S phase and is absolutely dependent on a functional MMR system.

Caffeine and UCN-01 abrogate the MMR-dependent G_2 arrest

We wanted to check whether the MNNG-induced cell cycle arrest observed in the 293T Lα$^+$ cells was brought about by a physical block to DNA synthesis (e.g., collapsed replication forks, aberrant recombination intermediates) or whether it was caused by the activation of a DNA damage checkpoint. As the latter process involves the major DNA damage-signaling protein kinases ATM and ATR, which are inhibited by caffeine (Sarkaria et al. 1999; Zhou et al. 2000), we decided to test whether the MNNG-induced arrest was sensitive to this drug. As shown in Figure 2, this was indeed the case. Fluorescence-activated cell sorting (FACS) analysis of cell populations doubly stained with propidium iodide and an antibody against the phosphorylated form of histone H3 (Xu et al. 2001) allowed us to distinguish between G_2-arrested and mitotic cells, as H3 is phosphorylated on Ser 10 only during mitosis (Crosio et al. 2002). In the initial set of experiments (data not shown), we pretreated the cells with caffeine 30 min before adding MNNG and then incubated the cells for a further 24 or 48 h. Using this protocol, we failed to observe any differences between caffeine-treated and untreated cells, as measured

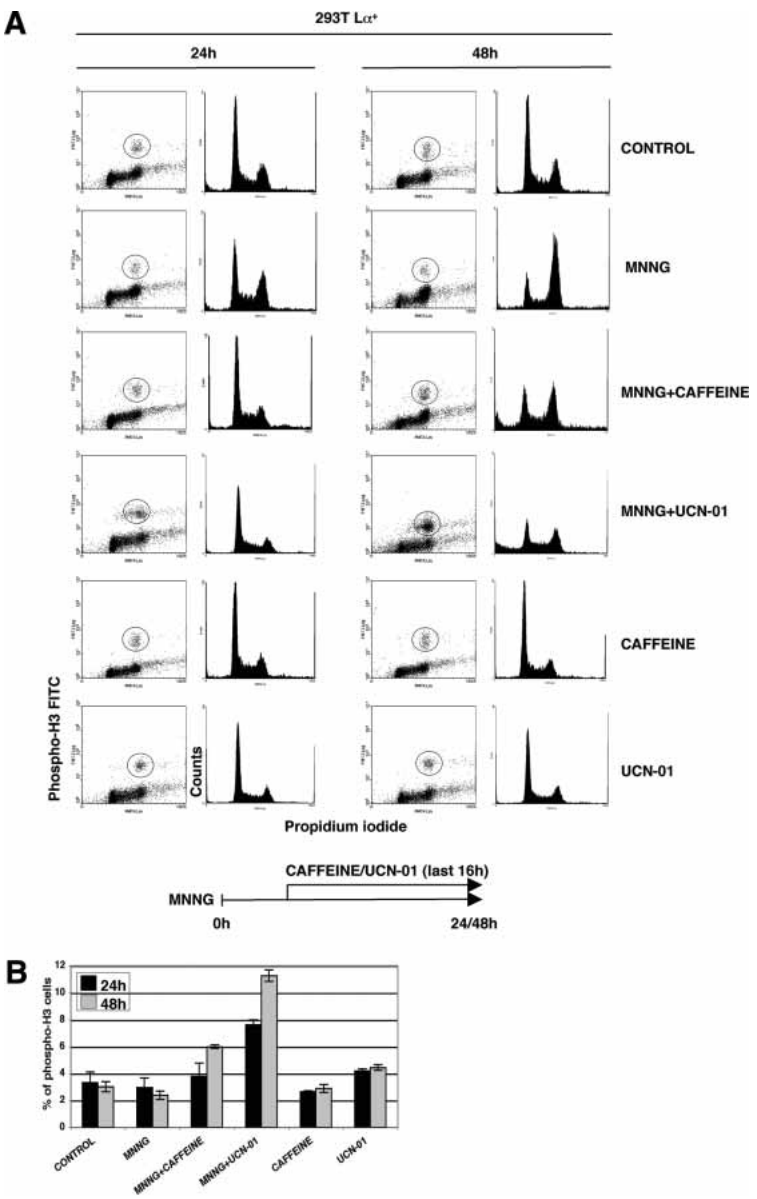

Figure 2. The G_2 arrest in MMR-proficient 293T Lα^+ cells is caffeine- and UCN-01-sensitive. (*A*) 293T Lα^+ cells were treated with MNNG (0.2 µM) for the indicated times, and caffeine (2 mM) or UCN-01 (100 nM) was added 16 h before harvesting. The cells were stained with propidium iodide (PI) and phospho-histone H3 antibody (cells in circle) to distinguish mitotic cells from those in G_2. The results show that both inhibitors attenuated the G_2 arrest in MNNG-treated cells. (*B*) Quantification of phospho-H3-positive cells from *A*. The number of cells entering mitosis in samples treated with MNNG and caffeine or UCN-01 was higher than in the controls, which shows that these kinase inhibitors abrogated the G_2 arrest and allowed more cells to enter mitosis.

by Western blotting with the phospho-H3 antibody, probably because the half-life of caffeine is only 4.5 h. We therefore added the kinase inhibitor some hours after the MNNG treatment. Using this protocol, MNNG-treated cells with a DNA content of 4n accumulated as observed previously (Fig. 1B), but the addition of caffeine to the treated cells 16 h before harvesting reduced the number of arrested cells by a substantial amount at the 24- and 48-h time points, as well as causing substantial cell death (Fig. 2A). That the latter effect was linked to an increased fraction of cells arriving in mitosis with unrepaired DNA is witnessed by a greater number of mitotic cells with phosphorylated histone H3 (Fig. 2B).

The initiation of G_2 arrest requires CHK1, the major transducer of ATR-dependent DNA damage signaling (Liu et al. 2000). As CHK1 kinase activity can be preferentially inhibited by the staurosporine analog UCN-01 (Busby et al. 2000; Graves et al. 2000), we studied the response of MNNG-treated cells to this drug in a way analogous to that deployed for caffeine. FACS analysis (Fig. 2A) showed that UCN-01 treatment abrogated the MNNG-induced G_2 checkpoint in 293T Lα^+ cells to an even greater extent than caffeine. Correspondingly, the fraction of cells arriving in mitosis, seen in FACS analysis as phospho-H3-positive cells, was higher in the MNNG- and UCN-01-treated samples than in cells treated with MNNG and caffeine (Fig. 2B).

Taken together, these results demonstrate that the MNNG-induced cell cycle arrest was indeed induced by a DNA damage-signaling cascade. Our data thus help explain the nature of the in *trans* inhibition of DNA replications in cells treated with methylating agents that was described more than a decade ago (Zhukovskaya et al. 1994).

Low-dose MNNG treatment brings about MMR-dependent phosphorylation of downstream targets of both ATM and ATR

ATM and ATR are both activated by DNA damage. However, whereas ATM responds rapidly to clastogenic damage such as that induced by IR (Bakkenist and Kastan 2003), ATR responds slower and cooperates with ATM in the later phases of the response (Brown and Baltimore 2003). ATR is also known to be preferentially activated on replication fork arrest induced by ultraviolet (UV) light, hydroxyurea (HU), or DNA polymerase inhibitors such as aphidicolin (Abraham 2001; Osborn et al. 2002). As MNNG treatment is thought to exert its cytotoxicity through the processing of ^{6Me}G residues during DNA synthesis (Karran and Bignami 1992), it might be anticipated that the damage-induced signaling cascade would initiate in S phase and involve ATR rather than ATM. Indeed, when the 293T Lα$^+$ cells were treated with 0.2 μM MNNG, phosphorylation of the ATR-activated checkpoint kinase CHK1 on Ser 345 became detectable after 12 h and peaked at 48 h, whereas phosphorylation of Thr 68 of CHK2, a preferred target for ATM, lagged by 12 h and increased steadily until 72 h (Fig. 3A).

We also examined the posttranslational modification of the single-stranded DNA-binding protein RPA, reported to redirect its function from replication to repair (Wang et al. 2001) through recruitment of the ATR/ATRIP (ATR-interacting protein) complex onto sites of

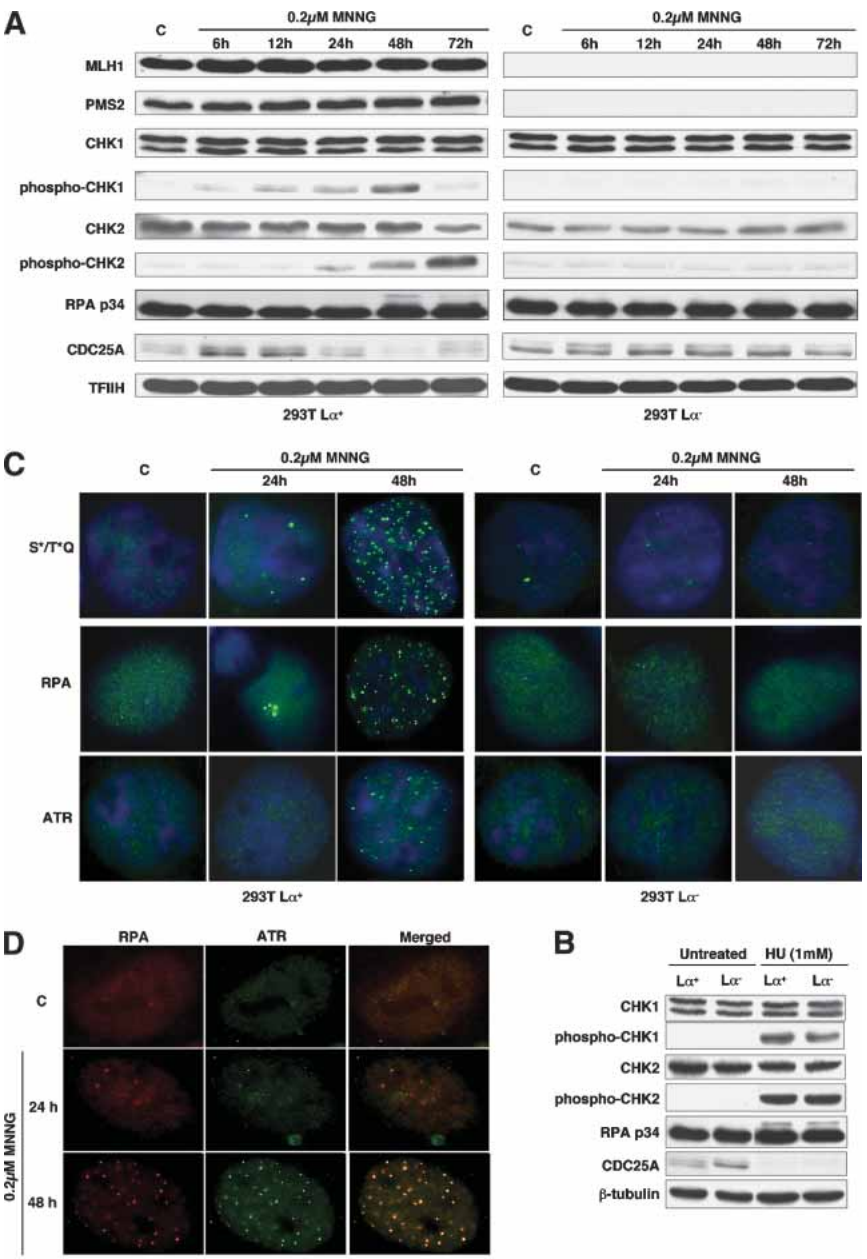

Figure 3. MMR-dependent DNA damage signaling in 293T Lα cells. (*A*) The 293T Lα$^+$ cells express hMLH1 and hPMS2 and are MMR proficient. Treatment with 0.2 μM MNNG brought about the phosphorylation of CHK1 and CHK2, as well as that of the single-strand DNA-binding protein RPA (p34), while CDC25A was gradually degraded. None of these modifications was observed in the MMR-deficient 293T Lα$^-$ cells. The phosphorylation status of RPA is indicated by the slower migration of the modified polypeptides through polyacrylamide gels (48-h time point). TFIIH was used as loading control. (*B*) Treatment of 293T Lα cells with 1 mM HU brought about a MMR-independent phosphorylation of CHK1, CHK2, and RPA (p34) and the degradation of CDC25A within 24 h. β-tubulin was used as loading control. (*C*) Indirect immunofluorescence imaging of nuclear foci formed by protein targets of the ATM/ATR kinases phosphorylated on serine and threonine residues in the SQ or TQ motifs, RPA (p34) and ATR. As shown, the foci formed only in the MMR-proficient 293T Lα$^+$ cells and were most numerous 48 h after treatment. (*D*) Indirect immunofluorescence imaging of nuclear foci formed by RPA (p34) and ATR in HeLa cells treated with 0.2 μM MNNG. The images were superimposed using Adobe Photoshop software. (C) Control, untreated cells.

DNA damage, which leads to an ATR-mediated activation of CHK1 (Zou and Elledge 2003). The p34 subunit of RPA was indeed phosphorylated after MNNG treatment, and the timing of its posttranslational modification coincided with the appearance of the highest levels of phosphorylated CHK1 (Fig. 3A).

The steady-state levels of CDC25A, a cell cycle phosphatase that is degraded on phosphorylation by CHK1 or CHK2 (Falck et al. 2001; Zhao et al. 2002), began to decline 24 h after treatment, at which time point only CHK1 kinase appeared to be activated. CDC25A controls the activation of CDK1 and CDK2 kinases and is known to regulate the G_1 (Hoffmann et al. 1994), intra-S (Falck et al. 2001), and G_2/M (Mailand et al. 2002) checkpoints. Its phosphorylation by CHK1/CHK2 leads to its destruction by the proteasome and thus also to cell cycle arrest. Indeed, 24 h after treatment, CDC25A levels were substantially lower than at the earlier time points. Taken together, this evidence suggests that ATR downstream targets were posttranslationally modified during the first cell cycle and that CHK2, a target of ATM, became activated later, after the second S phase. Importantly, none of these phenomena were apparent in the MMR-deficient 293T Lα$^-$ cells (Fig. 3A), which failed to arrest following MNNG treatment (Fig. 1A,E).

In a control experiment, we treated the 293T Lα cells with HU, which is known to bring about a cell cycle arrest in the first S phase after treatment. As shown in Figure 3B, CHK1, CHK2, and RPA-p34 phosphorylation was detectable already at the 24 h time point and, as anticipated, no differences were observed between the MMR-proficient and the MMR-deficient cells. CDC25A was undetectable in the treated cells at this time point, again irrespective of the cells' MMR status. We failed to observe MMR-dependent differences in phosphorylation patterns and CDC25A degradation also after 6 and 48 h (data not shown). These results both confirm that the 293T Lα$^-$ cells do not have defective checkpoint activating pathways and show that the signals triggering the HU- and MNNG-dependent G_2 checkpoints are different.

MNNG treatment induces ATM/ATR activation in vivo

We set out to seek evidence of the activation of ATM and ATR protein kinases in living cells. To this end, we employed the phospho-(Ser/Thr) ATM/ATR substrate (S*/T*Q) antibody that was raised against peptides carrying SQ or TQ amino acid motifs known to be posttranslationally modified by these kinases in several different substrates, and that is an accepted marker of ATM/ATR-dependent phosphorylation events (DiTullio et al. 2002). As shown in Figure 3C, foci of phosphorylated polypeptides began to appear after 24 h, but they were most numerous 48 h posttreatment, where they were visible in 67% of the cells. A similar phenomenon was observed also for RPA (78% of cells with foci) and ATR (75% of cells with foci). Again, these changes were observed exclusively in the MMR-proficient 293T Lα$^+$ cells. In MNNG-treated MMR-proficient HeLa cells, the ATR and RPA foci colocalized (Fig. 3D; Zou and Elledge 2003). Notably, the initial signs of checkpoint activation in the form of phosphorylation of CHK1 and degradation of CDC25A in 293T Lα$^+$ cells (Fig. 3A) preceded the appearance of the foci in both cell types by ~40 h. This implied that the ATM/ATR kinases were activated long before the ATR, RPA, and S*/T*Q proteins formed the foci. As the appearance of the foci coincides with the formation of chromosomal aberrations (N. Mojas, L. Stojic, and J. Jirivny, in prep.), it is possible that the nuclear foci represent recombination intermediates arising during the second S phase.

ATM is dispensable for cell cycle arrest induced by low dose MNNG treatment

As shown above, the MNNG-induced G_2 checkpoint was released by UCN-01, which inhibits preferentially the CHK1 kinase, the preferred target of ATR. Given that the latter kinase has been implicated in the control of S-phase checkpoints triggered by arrested replication forks (Abraham 2001; Osborn et al. 2002) and that the nonproductive processing of ^{6Me}G/T mispairs by the MMR system should also signal during the S phase, it seemed logical that ATR also should be involved in the control of the MNNG-induced checkpoint described above. However, as ATR and ATM display a certain degree of functional redundancy, we wanted to exclude the involvement of the latter kinase in checkpoint activation. Under normal conditions, ATM is present in the nucleus in an inactive, dimeric form, but it can be rapidly activated by stress stimuli. This process involves disruption of the dimer and is accompanied by ATM autophoshorylation of Ser 1981 (Bakkenist and Kastan 2003). Using a specific antibody against this phosphorylated isoform, we were able to follow activation of the ATM kinase in the 293T Lα cells following treatment with 0.2 μM MNNG. In a control experiment, ATM was efficiently activated by IR treatment, irrespective of the MMR status of the cells, whereas HU treatment was significantly less effective in activating ATM, as anticipated (Fig. 4A). Treatment with MNNG resulted in ATM activation, although only at the 48- and 72-h time points, which coincided with the peak of phosphorylation of CHK2, a known downstream target of ATM (Fig. 3A). Notably, both these events were observed exclusively in the MMR-proficient 293T Lα$^+$ cells.

Although the above experiment demonstrated that ATM was activated in a MMR-dependent manner by MNNG treatment in 293T Lα$^+$ cells, it failed to show whether this kinase was indispensable for activation of the cell cycle arrest. This question was answered with the help of a matched pair of ATM-positive and ATM-negative fibroblast lines (Ziv et al. 1997), which displayed no major differences in G_2 arrest efficiency on MNNG treatment, as assessed by FACS analysis (Fig. 4B).

Consistent with the above evidence, the number and kinetics of appearance of S*/T*Q foci on treatment with

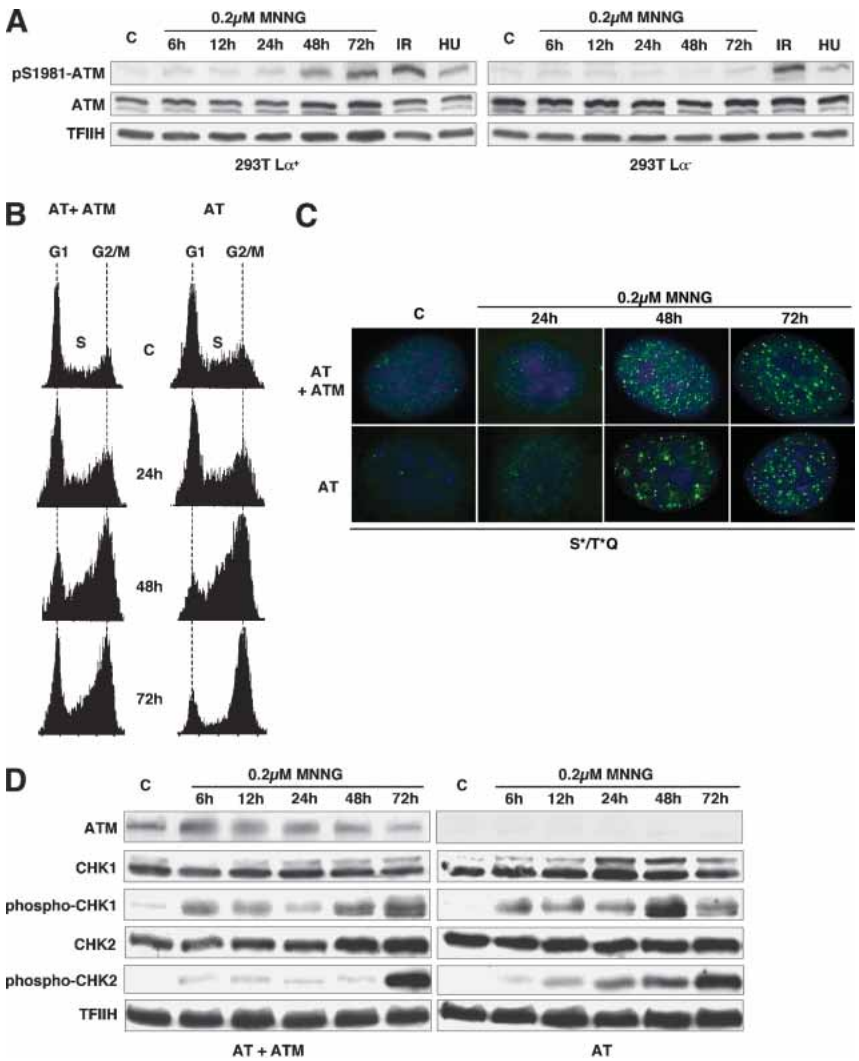

Figure 4. ATM is activated but dispensable for the MNNG-induced G_2 arrest in MMR-proficient cells. (*A*) ATM was activated early in both 293T Lα$^+$ and 293T Lα$^-$ cells on ionizing radiation (10 Gy, 1 h) and, to a lesser degree, after HU treatment (1 mM, 6 h). In contrast, on treatment with MNNG, ATM was activated late, and only in MMR-proficient (293T Lα$^+$) cells. ATM activation was assessed using an antibody against phosphorylated Ser 1981. (*B*) FACS analysis of unsynchronized cultures of AT and AT + ATM fibroblasts following treatment with 0.2 μM MNNG. Both ATM-proficient (AT + ATM) and ATM-deficient (AT) cells proceeded through the cell cycle with similar kinetics and began to accumulate in G_2/M after 2 d. (*C*) Indirect immunofluorescence imaging of nuclear foci formed by protein targets of the ATM/ATR kinases phosphorylated on serine and/or threonine residues in the SQ or TQ motifs. As shown, the foci began to form in both ATM-proficient and ATM-deficient cells after the 24 h time point. At 48 h, both cell types contained foci, even though they were less numerous in the AT cells. However, at 72 h, no significant differences in focus number or intensity were observed in the two cell types. (*D*) MNNG treatment leads to ATM-independent CHK1 and CHK2 activation, albeit with different kinetics.

0.2 μM MNNG was similar in the AT and AT + ATM cells (Fig. 4C). These foci were not detected in the AT cells on IR treatment (DiTullio et al. 2002), which strongly suggested that the lesions generated by the MMR system during processing of MNNG-induced damage are distinct from IR-induced strand breaks.

Analysis of protein phosphorylation cascades by Western blotting revealed that CHK1 and CHK2 were post-translationally modified in both cell lines, albeit with different kinetics (Fig. 4D). In a recent report, Wang and colleagues (2003) showed that in IR-treated AT-deficient cells, the ATR kinase compensated for the lack of ATM through overactivation of CHK1. We now extend these findings to MNNG treatment, as the phosphorylation of CHK1 at the 48-h time point was substantially stronger in the AT cells than in the corrected AT + ATM line. Taken together, the results presented in Figure 4 demonstrate that although MNNG treatment led to activation of ATM, this kinase was dispensable for triggering the protein phosphorylation cascade and the G_2 cell cycle arrest.

The MNNG-induced G_2 arrest and DNA damage-dependent signaling requires ATR

As ATM was not required for the MNNG-induced checkpoint activation (Fig. 4), we set out to confirm the involvement of ATR. Unlike in the case of ATM, there are no stable ATR-defective cell lines, as the loss of this kinase is lethal, and we therefore had to resort to using U2OS cells, which overexpress a kinase-dead variant of ATR (ATR-kd) under the control of the Dox-regulated TetOn expression system (Nghiem et al. 2002). ATR-kd overexpression was shown to abrogate the G_2 arrest (Cliby et al. 2002) and to sensitize cells to several DNA damaging agents (Nghiem et al. 2002), and we wanted to see how it affected the cellular response to MNNG. The U2OS cells were substantially more resistant to MNNG than the 293T Lα$^+$, AT, and ATM + AT lines (even though the MGMT activity of all the lines was inhibited with O^6-benzylguanine), and we therefore had to use a 1.5-μM concentration of the drug to obtain cytotoxicity comparable to that exerted on the latter cells by 0.2 μM

MNNG. Under these experimental conditions, the uninduced U2OS cells were largely arrested in the G_2 phase of the cell cycle 48 h after treatment (Fig. 5A, left panel), similar to the 293T Lα$^+$ cells (Fig. 1B). However, this arrest was substantially attenuated when the cells were induced to overexpress ATR-kd (Fig. 5A, right panel). Phosphorylation of CHK1, seen in the uninduced cells, was totally abrogated by overexpression of ATR-kd, whereas CHK2 phosphorylation remained largely unchanged (Fig. 5B). Moreover, overexpression of ATR-kd had a dramatic effect on the formation of S*/T*Q foci (Fig. 5C,D). The uninduced cells displayed no defect in focus formation: Both RPA and S*/T*Q foci were abundant 48 h after MNNG treatment, and the fact that they largely colocalized substantiated recent reports that demonstrated the requirement for RPA-bound stretches of single-stranded DNA for the recruitment of ATR and for focus formation (Zou and Elledge 2003). In ATR-kd overexpressing cells, the RPA foci formed earlier, but we failed to observe foci of S*/T*Q. This demonstrated that the kinase activity of ATR is required for the formation of the latter foci. This experimental evidence also demonstrated that the formation of RPA foci is ATR independent (i.e., that RPA is recruited to sites of damage before ATR, as discussed by others [Zou and Elledge 2003]).

MNNG treatment induces formation of γ-H2AX foci that are not associated with double-strand breaks, but colocalize with the foci of RPA

We wanted to gain information about the type of damage generated by the MMR-dependent processing of MNNG-induced DNA damage. Using alkaline comet assays, we found evidence of extensive single-stranded DNA degradation in both MMR-deficient and MMR-proficient cells already 4 h posttreatment. Under these conditions, apurinic sites generated by removal of methylated bases are cleaved and the strand breaks become detectable as the double-stranded DNA is denatured. Importantly, the single-strand breaks completely disappeared with time in the MMR-deficient cells, whereas in the MMR-proficient cells, a substantial proportion persisted even 48 h after treatment (Fig. 6A,B). On the basis of this evidence, we expected to observe no nuclear foci of the phosphorylated form of histone H2AX (γ-H2AX), which was re-

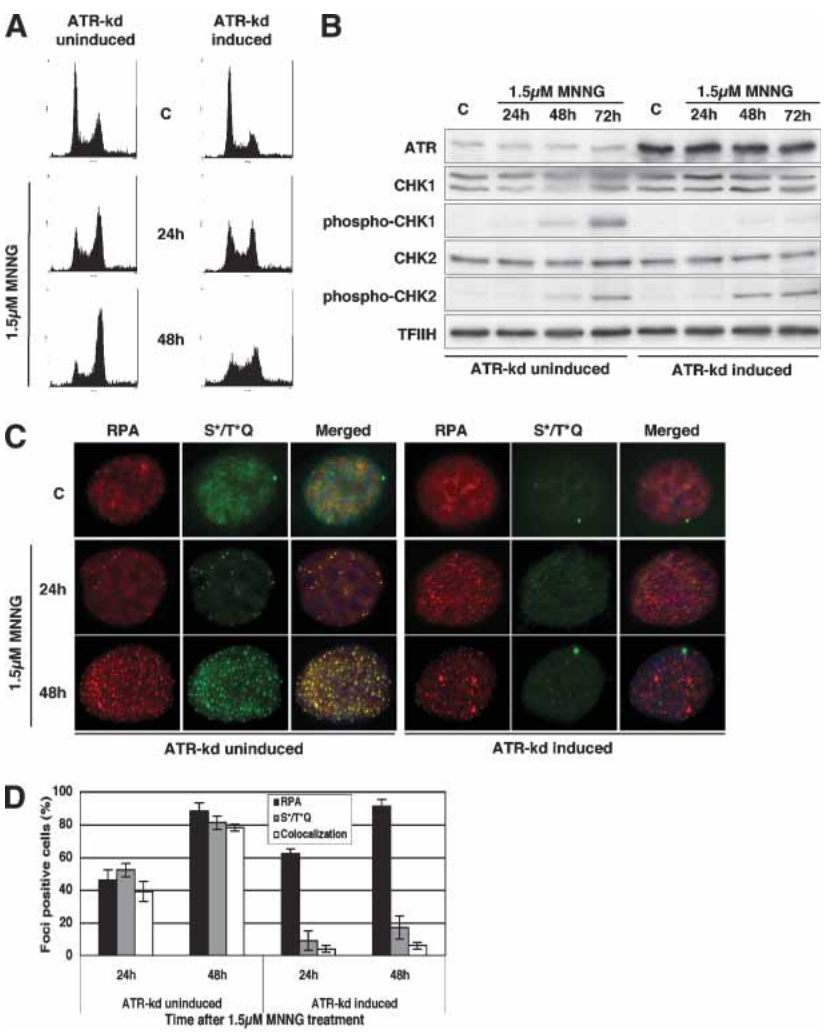

Figure 5. The G_2 checkpoint induced by low MNNG doses is ATR-dependent. (*A*) FACS analysis of U2OS cells that overexpress the kinase-dead ATR variant under doxycycline control. The figure shows that the G_2 arrest activated by MNNG treatment in these cells was attenuated by overexpression of the ATR-kd protein. (*B*) CHK2 phosphorylation was largely unaffected by overexpression of ATR-kd in the treated U2OS cells. In contrast, activation of CHK1 was dependent on the presence of functional ATR. (*C*) Indirect immunofluorescence of ATR-kd inducible U2OS cells showing that formation of S*/T*Q foci and their colocalization with RPA (p34) after MNNG treatment is ATR-dependent. The fraction of foci-displaying cells on each microscope slide is shown in panel *D*.

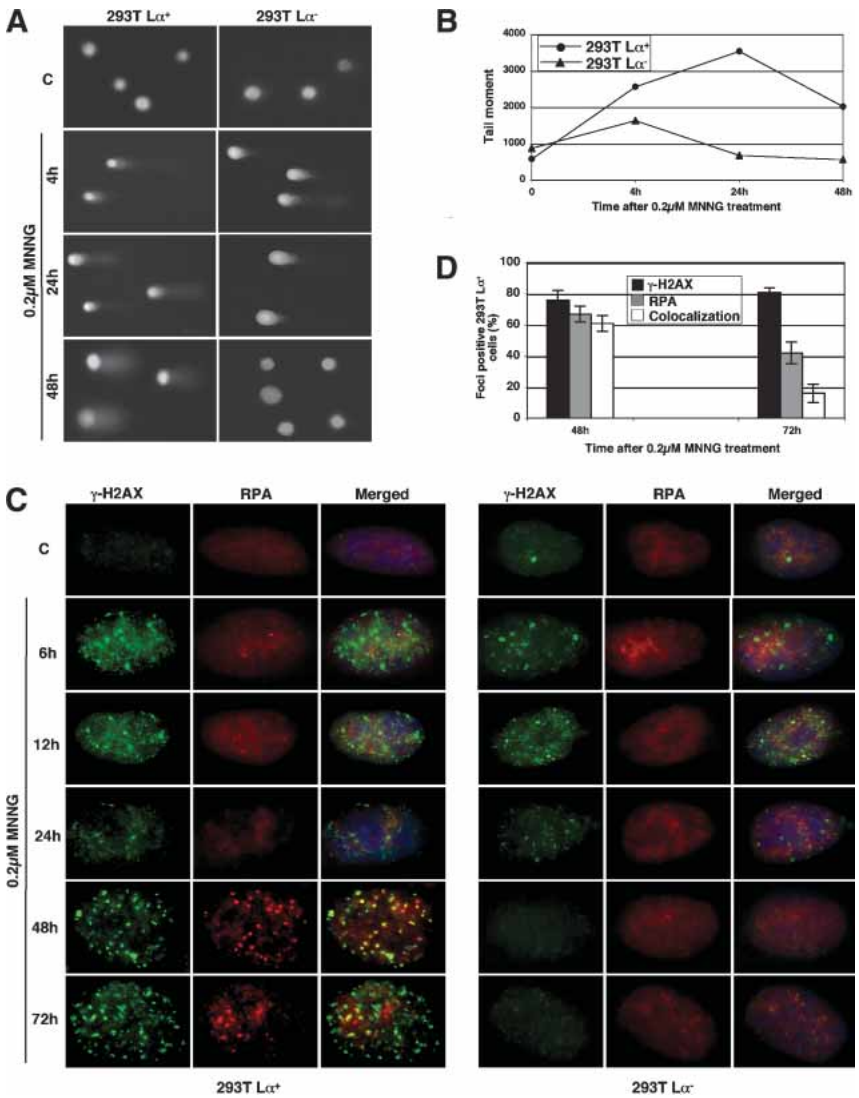

Figure 6. MMR-dependent processing of methylation damage. (*A*) Alkaline comet assays showing the appearance and repair of DNA single-strand breaks in 293T Lα cells on 0.2 μM MNNG treatment. The panel shows representative cells. (*B*) Quantification of the tail moment of 50 randomly selected cells per slide. As shown, the single-strand breaks (or gaps) were repaired in the MMR-deficient cells but persisted in the MMR-proficient ones. (*C*) Kinetics of histone H2AX phosphorylation and the colocalization of γ-H2AX foci with those of RPA in MNNG-treated 293T Lα cells. The γ-H2AX foci appeared soon after treatment, independent of the MMR status of the cells. They then diminished in number in both cell types but began to reappear in the MMR-proficient 293T Lα$^+$ cells after 24 h. At the 48-h time point, they largely colocalized with the foci of RPA, but this overlap diminished by 72 h. In contrast, in the MMR-deficient 293T Lα$^-$ cells, the γ-H2AX foci disappeared completely. The panel shows representative cells. The fraction of cells on each microscope slide displaying a similar phenotype is shown in *D*.

ported to associate with DSBs (Rogakou et al. 1999) and to recruit repair factors to these sites (Paull et al. 2000). Surprisingly, numerous γ-H2AX foci appeared soon after MNNG treatment (Fig. 6C). The lesions associated with these early foci were apparently not responsible for triggering the signaling cascade, as they appeared in similar numbers and with similar kinetics in both MMR-proficient and MMR-deficient cells and as no signaling that could be ascribed to DSBs was detected. Moreover, when the treated cells were examined by pulse field gel electrophoresis, TUNEL, and neutral pH comet assays, no DSBs could be detected (data not shown). It is therefore highly unlikely that the early-appearing γ-H2AX foci seen in this study represent sites of DSB formation. A more likely scenario is that they represent regions in which chromatin structure is disrupted because of the processing of modified purines by the BER system.

At later time points, the γ-H2AX foci gradually disappeared from the nuclei of MMR-deficient cells, while in the MMR-proficient cells they appeared to change morphology and increased in number and intensity. We wanted to test whether these foci colocalized with those formed by RPA, given that the single-stranded DNA-binding protein was seen to colocalize with ATR and the S*/T*Q substrate (Figs. 3D, 5C, respectively). As shown in Figure 6C and D, the foci of γ-H2AX and RPA were seen to colocalize in ~60% of the treated cells at the 48-h time point. At the 72-h time point, the intensity of the RPA foci diminished and very little colocalization with the γ-H2AX foci could be seen.

Discussion

A functional MMR system has been postulated to be required for the activation of a G_2/M cell cycle arrest (Hawn et al. 1995; Claij and Te Riele 2002; Cejka et al. 2003) and for apoptosis (D'Atri et al. 1998) induced in mammalian cells by S_N1 type methylating agents and 6-thioguanine. Using an isogenic system developed in our laboratory (Cejka et al. 2003), in which the MMR status of the 293T Lα cells can be controlled by Dox, we show that the MMR-proficient cells treated with 0.2 μM

MNNG arrested in the G_2 phase of the second cell cycle (Fig. 1A,B), rather than undergoing a mitotic catastrophe (Fig. 1C). This was highly unusual, because cells generally arrest a few hours after DNA damage. We therefore set out to identify the molecular mechanisms underlying this phenomenon. In the first series of experiments, we showed that the accumulation of MNNG-treated cells in G_2 was attenuated by caffeine and UCN-01, which are known to inhibit preferentially the ATM/ATR and CHK1 kinases, respectively (Fig. 2). This evidence further confirmed that the increase in the number of cells with a 4n DNA content, as observed by FACS, was the result of activation of a checkpoint, rather than of a physical block imposed by the DNA damage. Correspondingly, we could show that MNNG treatment of the MMR-proficient cells activated a protein phosphorylation cascade that modified a number of downstream targets of the ATM/ATR kinases (Fig. 3). It appeared most likely that these phosphorylation events actually triggered the arrest, as the posttranslational modification of these targets temporally coincided with its activation. We were able to rule out the requirement for ATM in the activation of the MNNG-induced checkpoint: Although the kinase appeared to be activated at late time points in the MNNG-treated 293T Lα^+ cells (Fig. 4A), the AT fibroblasts lacking this kinase arrested similarly to ATM-proficient ones (Fig. 4B). This hypothesis is further supported by earlier findings, which showed that HCT15 cells that lack CHK2, one of the downstream targets of ATM, arrested normally on treatment with methylating agents when their MMR defect was corrected (Umar et al. 1997). In contrast, ATR kinase and its downstream target CHK1 were shown to be required for the efficient activation of the MNNG-induced checkpoint, as the number of cells with a 4n DNA content was dramatically decreased in MNNG-treated U2OS cells overexpressing the kinase-dead ATR variant (Fig. 5), or in the 293T Lα^+ cells when the CHK1 activity was inhibited by UCN-01 (Fig. 2).

Our finding that the ATM kinase is activated only very late after MNNG treatment seemingly contrasts with the data of Adamson and colleagues (2002), who reported that MNNG treatment rapidly activates this enzyme. These differences are probably the result of the 120-fold higher dose of the reagent used in the latter study. High concentrations of DNA methylating agents bring about levels of base damage that are too high to be effectively processed by the BER system. As a result, strand breaks arising through incomplete BER appear soon after treatment and activate damage-signaling pathways that are more reminiscent of those induced by other clastogenic DNA damaging agents. Importantly, this processing is largely independent of the MMR system (L. Stojic, N. Mojas, P. Cejka, and J. Jiricny, in prep.).

The involvement of damage-specific kinases other than ATM and ATR in the MNNG-induced cell cycle arrest has not been ruled out. However, it is unlikely that DNA-dependent protein kinase (DNA-PK) plays a key role, as it generally does not appear to be required for DNA damage signaling (Durocher and Jackson 2001). Moreover, cells mutated in its Ku80 subunit are hypersensitive to IR but appear to respond normally to MNNG (Jeggo and Kemp 1983).

If ATR is the most upstream DNA damage-signaling kinase, what is the nature of the MNNG-induced lesions that trigger its activation? Our results show that the kinase cascade is not activated directly by ^{6Me}G/T mispairs (e.g., through interaction with the mismatch binding heterodimer hMSH2/hMSH6; Duckett et al. 1996; Fishel 1999). First, these mispairs should have already arisen during the first S phase, and even if they were to activate the signaling cascade directly, there is no reason why cells should be arrested in the second cell cycle, when the number of these mispairs is reduced by 50% because of the semiconservative nature of DNA replication. (The half-life of MNNG in culture medium is ~1 h; it has thus been inactivated long before the onset of the second cell division.) Second, cells lacking hMLH1/hPMS2 (e.g., HCT116, 293T, 293T Lα^-) express normal levels of the hMSH2/hMSH6 heterodimer, yet are also highly resistant to killing by MNNG and do not arrest in G_2. This would require that the DNA damage signaling be mediated by the hMSH2/hMSH6/hMLH1/hPMS2 heterotetramer. Although formally possible, the appearance of RPA foci suggests that the signaling was initiated through processing rather than just detection of the damage—but what is the nature of this processing?

More than 30 years ago, Plant and Roberts (1971) suggested that replication past ^{6Me}G in the template strand during the first S phase may give rise to single-stranded gaps, which are converted into DSBs during the second replication cycle. This was long thought to be unlikely, as DNA polymerases were expected to repair gaps remaining from incomplete replication during the G_2 phase. However, it is conceivable that such gaps do indeed arise in DNA methylated by S_N1-type agents. During DNA replication, the polymerases may incorporate a T or a C into the newly synthesized strand opposite the ^{6Me}G residues in the template strand, and it has been suggested that the MMR system will detect these non-Watson-Crick structures (Duckett et al. 1996) and attempt to repair them. The repair process would exonucleolytically degrade a short stretch of the newly replicated DNA (i.e., the strand containing the pyrimidines). However, as the ^{6Me}G residues persist in the template strand, resynthesis of this region would again generate ^{6Me}G/T or ^{6Me}G/C mispairs. The repeated processing of these mispairs by the MMR system (Karran and Bignami 1996) will likely lead to stalling of the replication fork. One might pose the question of why these structures would fail to activate the S-phase checkpoint, when other polymerase-arresting agents such as HU or aphidicolin do so extremely efficiently (Abraham 2001; Osborn et al. 2002; Shiloh 2003). One possible explanation is that HU and aphidicolin inhibit all active replicons, whereas the number of ^{6Me}G residues generated by MNNG treatment may be too low to trigger an S-phase arrest. An alternative explanation is that unlike HU, which brings about a depletion of the purine nucleotide pool, or aphidicolin, which directly inhibits the replica-

tive polymerases, ^{6Me}G residues in the template strand do not prevent a replication restart downstream from the modified base. Indirect support for the replication restart hypothesis comes from in vitro experiments carried out with MNU-modified plasmid DNA; the observed DNA repair synthesis required nucleoside triphosphates (NTPs), which would be required by a primase (Ceccotti et al. 1993, 1996). Furthermore, these experiments demonstrated that the repair synthesis triggered by the presence of ^{6Me}G residues in the DNA is RPA independent and that it gives rise to open circular DNA, in contrast to the in vitro replication reaction of the same, but unmodified, plasmid, which yielded almost exclusively supercoiled DNA molecules. These results could be taken as further evidence of the persistence of unligatable single-stranded regions or breaks in the plasmid DNA incubated with MMR-proficient cell extracts (Ceccotti et al. 1996). Direct support for the persistence of incompletely replicated DNA comes from our experiments, in which the genomic DNA of MNNG-treated MMR-proficient cells after the first S phase was shown to contain numerous single-strand breaks, as witnessed by the appearance of DNA tails in alkaline comet assays (Fig. 6A,B). Our findings are further supported by recent evidence showing that treatment of cells with 6-thioguanine, which is believed to exert its cytotoxicity via a mechanism analogous to MNNG (Swann et al. 1996), also results in the accumulation of MMR-dependent single-strand DNA breaks (Yan et al. 2003).

The latter hypothesis raises two important questions. First, if genomic DNA containing ^{6Me}G residues does indeed contain single-strand gaps after the first S phase, why do such gaps not activate the checkpoint already at this time? One possibility is that they are too few in number. Alternatively, the gaps might not stall the replisome, or they might be too short to be bound by RPA. Indeed, the RPA foci began to appear only at 24 h, and their number peaked at the 48-h time point (Figs. 3C,D, 5C,D). As RPA has been shown to be required for the efficient recruitment of the ATR/ATRIP complex to the sites of damage, single-strand gaps that are not RPA bound would fail to efficiently activate the CHK1 kinase, which has been identified in complexes that associate with strand breaks and with single-stranded DNA (Goudelock et al. 2003) and which was shown to be involved in the MNNG-induced G_2 checkpoint (Fig. 2).

Second, assuming that the single-stranded gaps do indeed form, how could they persist until the subsequent S phase as suggested (Plant and Roberts 1971; Kaina et al. 1997)? This could be the result of a combination of factors. As discussed above, it is possible that, in the absence of bound RPA, the damage sites may signal too weakly to effectively activate the checkpoint. The other reason might be that the filling of a gap opposite a ^{6Me}G residue may not be trivial. Thus, proliferating cell nuclear antigen (PCNA)-dependent polymerases tested to date would generate ^{6Me}G/C or ^{6Me}G/T mispairs (Reha-Krantz et al. 1996), which would be again addressed by the MMR system, because of its ability to interact with the processivity factor (Kleczkowska et al. 2001). Other polymerases might have problems extending from the non-Watson-Crick ^{6Me}G/C or ^{6Me}G/T structures, in which case the DNA synthesis would stall at the mispairs because of the activation of the $3' \rightarrow 5'$ proofreading activity (Khare and Eckert 2001).

Persistent single-strand gaps opposite the ^{6Me}G residues would become DSBs during the second S phase, and the affected replication forks would collapse unless rescued by recombination events such as sister chromatid exhanges (SCEs). That events of this type indeed arise in cells treated with methylating agents was suggested by an increase in SCE frequency in the treated MMR-proficient 293T Lα^+ cells (data not shown; N. Mojas, L.Stojic, and J. Jiricny, in prep.; see also Galloway et al. 1995; Kaina et al. 1997). The timing of these events broadly coincided with the formation of foci containing RPA, ATR (Fig. 3C,D), and γ-H2AX (Fig. 6C), which may represent the sites in chromatin at which processing is taking place and also from which the signaling events may be originating.

In conclusion, treatment of mammalian cells with a low dose of MNNG was shown to bring about a G_2 cell cycle arrest that was absolutely dependent on a functional MMR system and that, to a substantial degree, was also dependent on the ATR and CHK1 kinases. This checkpoint was highly atypical, inasmuch as it came into effect only in the second cell cycle after treatment. Its activation was accompanied by a number of changes in the nuclei of the treated cells, possibly indicative of recombination events. Our present findings thus suggest that S_N1 type methylating agents such as the chemotherapeutics procarbazine and temozolomide, which act similarly to MNNG, kill cells with the help of MMR, which generates intermediates that cannot be effectively processed by the cellular repair machinery. The persistence of these lesions into the second cell cycle may kill cells through the generation of aberrant recombination intermediates. We are currently attempting to elucidate the structures of these lesions by biophysical techniques.

Materials and methods

Cell lines

The 293T Lα cell line was established in our laboratory and propagated as described (Cejka et al. 2003). HeLa cells were maintained in DMEM (OmniLab) supplemented with 10% fetal calf serum (FCS; Life Technologies), penicillin (100 U/mL), and streptomycin (100 µg/mL). The ATM-deficient (AT) fibroblasts AT22IJE-T and the matched line complemented with ATM minigene (AT + ATM) were kindly provided by Yosef Shiloh (Tel Aviv University, Israel) and were maintained as described (Ziv et al. 1997). The U2OS cell line conditionally expressing ATR kinase-dead protein (Paul Nghiem, Harvard University, Cambridge, MA) was maintained in DMEM supplemented with 10% FCS, 200 µg/mL G418, and 200 µg/mL Hygromycin B. Induction of ATR-kd was accomplished by supplementing the growth medium with Dox (1 µg/mL) for 48 h, as described (Nghiem et al. 2002). The hMLH1-deficient human colon cancer cell line HCT116 and its MMR-proficient subline HCT116 + ch3 were maintained in McCoy's 5A medium (OmniLab) with 10% FCS.

The chromosome-complemented cell line was maintained in medium containing 400 µg/mL G418. Expression of all MMR proteins was confirmed in both AT fibroblasts and ATR-inducible cells by immunobloting (data not shown). To inhibit MGMT activity, HeLa cells, HCT116 and HCT116 + ch3 cells, AT and AT + ATM fibroblasts, and the ATR-kd-inducible cells were pretreated with 10 µM O^6-benzylguanine 2 h before MNNG treatment. All cell lines were cultured at 37°C in a 5% CO_2 humidified atmosphere.

Chemicals and irradiations

MNNG (Sigma) was dissolved in DMSO and stored at –20°C in the dark. O^6-benzylguanine (Sigma) was dissolved in ethanol and stored at –80°C. HU (Sigma) and Dox (Clontech) were dissolved in water and stored at –20°C. UCN-01 (Sally Hausman, Cancer Therapy Evaluation Program, National Cancer Institute, National Institutes of Health, Rockville, MD) was dissolved in DMSO and stored at –20°C. Caffeine (Calbiochem) was dissolved in water and always prepared fresh. Irradiations were carried out at the doses indicated, using a Philips PW2184/00-Monitor SN4.

Mitotic index assays

The 293T Lα cells were treated with 0.2 µM MNNG and incubated for 24 or 48 h. Nocodazole (0.3 µg/mL, Sigma) was then added and the cells were incubated for a further 24 h. The floating and attached cells were then harvested and centrifuged at 400*g*. The pellet was suspended in 3 mL of 75 mM KCl for 10 min, centrifuged again as above, and resuspended in Carnoy's fixative (1:3 v/v acetic acid:methanol). This latter step was repeated three times. Twenty microliters of the cell suspension were spotted onto a microscope slide and allowed to air dry. Once dry, the cells were stained with 0.1 µg/mL 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI; Sigma) for 10 min, washed with water, and mounted in SlowFade Antifade (Molecular Probes). Using a fluorescence microscope, cells with broken nuclei and condensed chromatin were counted as mitotic. Five hundred cells were counted per sample.

Cell synchronizations

The 293T Lα cells were grown to 50% confluency in a serum-rich medium. Thymidine (2 mM, SynGen Inc.) was added, and the cells were incubated for 18 h, washed three times with PBS, and released into thymidine-free medium for 9 h. Thymidine (2 mM) was then added for a further 15 h. The cells were then washed three times with PBS. At this point (G_1/S, Fig. 1A), the cells were treated with 0.2 µM MNNG in a serum-rich medium without thymidine, and time points were collected 4 (T 4), 8 (T 8), 14 (T 14), 20 (T 20), 24 (T 24), and 30 (T 30) h later. HCT116 and HCT116 + ch3 cells were synchronized in a medium containing 2 mM HU for 14 h. O^6-benzylguanine (10 µM) was added for the last 2 h, when the cells were washed with PBS. The cells were then incubated in fresh medium containing 0.2 µM MNNG (G_1/S, Fig. 1B) and O^6-benzylguanine. Cells were harvested and analyzed by propidium iodide-flow cytometric analysis as described (Cejka et al. 2003).

Cell cycle analyses

For BrdU labeling, cells were pulse-labeled with 10 µM BrdU (Sigma) for 30 min before harvesting and fixation in 70% ethanol at 4°C. The cells were then processed as described (Cliby et al. 2002). BrdU incorporation studies and cell cycle distributions were analyzed by Becton Dickinson CELLQuest software. For immunofluorescence-based detection of phosphorylated histone H3, the cells were treated with 0.2 µM MNNG. Sixteen hours before harvesting, the growth medium was supplemented with caffeine (2 mM) or UCN-01 (100 nM). The cells were collected 24 or 48 h after MNNG treatment. The subsequent steps were carried out as described (Xu et al. 2001).

Cell doubling assays

Cell doubling assays were carried out as described previously (Cejka et al. 2003).

Alkaline comet assays

Alkaline comet assays were carried out using Trevigen CometSlides according to the manufacturer's recommendations. DNA was stained with ethidium bromide (10 µg/mL) and visualized using a fluorescence microscope. Fifty comets were analyzed per slide, using National Institutes of Health images with Comet macro (Helma and Uhl 2000).

Antibodies and immunoblotting

Anti-MLH1 (554072) and anti-PMS2 (556415) monoclonal antibodies were from BD Pharmingen; anti-CHK1 (611152) was from BD Transduction Laboratories; and anti-β-tubulin (D-10), anti-TFIIH p89 (S-19), anti-CDC25A (F-6), and anti-ATR (FRP1, N-19) were from Santa Cruz Biotechnology. Anti-RPA p34 (Ab-3) was from Oncogene. Anti-phospho-CHK1 (Ser 345), anti-phospho-CHK2 (Thr 68), and anti-phospho-Ser/Thr (S*/T*Q) ATM/ATR substrate antibodies were from Cell Signaling. Anti-CHK2 (07-126) and anti-γ-H2AX (Ser 139) antibodies were from Upstate Biotechnology. The anti-ATM phospho-Ser 1981 antibody was obtained from Rockland. The anti-ATM antibody was kindly provided by Stephen P. Jackson (Wellcome/CRC Institute, Cambridge, UK). Immunoblotting and total protein extractions were performed as described previously (Cejka et al. 2003).

Immunofluorescence studies

Cells grown on glass cover slips were treated or mock-treated with MNNG and incubated for the indicated time periods. Fixation was done in 3.7% formaldehyde/PBS (15 min, 4°C), followed by permeabilization in 0.2% Triton X-100/PBS (5 min, 4°C). In the case of anti-γ-H2AX, the cells were fixed in ice-cold methanol (20 min, –20°C). The coverslips were blocked with 3% low-fat milk/PBS and incubated with anti-phospho-(Ser/Thr) ATM/ATR substrate, anti-γ-H2AX (Ser 139), anti-ATR, and anti-RPA p34 antibodies, all at 1:100 dilution. After washing, the cells were incubated with FITC-conjugated anti-rabbit or anti-goat antibodies (Sigma) and TR-conjugated anti-mouse antibodies (Abcam) for 1 h at 37°C. The nuclei were counterstained with DAPI (0.1 µg/mL, Sigma). Images were captured on a Leica DC 200 fluorescence microscope.

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4.3 Recombinational processing of MMR-induced DNA lesions created by S_N1-type methylating agents

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In order to better understand the mechanism of cell killing by MNNG, I wanted to understand what are the pathways required for MMR-dependent processing and how do they contribute to cell death or survival. I was able to show that the initial recognition of ^{6me}G-containing mispairs by MMR happened in the first S phase after treatment and this provoked cytologically visible uncoupling of replication and repair foci. Although recognized in the first cell cycle, in order to induce cell cycle arrest, presence of ^{6me}G is required in both cell cycles after MNNG treatment indicating involvement of another pathway. Activation of the MRN complex was MMR dependent, but not necessary for the arrest or overall survival. *In vivo* activation of homologous recombination depended on MMR status and the sites of ongoing recombination contained the ssDNA-binding protein RPA. Lack of HR repair rendered cells extremely sensitive to MNNG, and activated cell cycle arrest already in the first cycle after treatment. This finding suggests that progression through the first cell cycle requires homologous recombination, possibly to rescue the intermediates created by MMR processing after ^{6me}G mispairing in the first replication.

Recombinational processing of MMR-induced DNA lesions created by S_N1-type methylating agents

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Abstract

The cytotoxicity of methylating agents of S_N1-type, such as *N*-methyl-*N'*-nitro- *N* -nitrosoguanidine (MNNG), has been proposed to be mediated by unsuccessful attempts of the mismatch repair (MMR) system to process mispairs between O^6-methylguanine (^{6me}G) and cytosine or thymine, which arise during DNA replication. This hypothesis is based on data showing that cells resistant to killing by these agents either express high levels of methylguanine methyl tranferase (MGMT), an enzyme that selectively reverts ^{6me}G to G, or have defective MMR. However, this hypothesis fails to explain why the MNNG-induced cell cycle arrest occurs only in the second G_2 phase after treatment. In an attempt to elucidate this phenomenon, we set out to identify the DNA metabolic pathways that transform ^{6me}G-containing mispairs into cytotoxic lesions. We now show that the mismatch recognition factor MutSα localises to replication foci in the first S phase after MNNG treatment of MGMT-depleted cells, but that the MutSα foci persist long after replication has ceased. Reactivation of MGMT after the first cell cycle abolished the cell cycle arrest, which shows that persistent ^{6me}G residues are required for its activation. In contrast, activation of the MRE11/NBS1/RAD50 (MRN) complex, required in the repair of double-strand breaks, was dispensable for cell cycle arrest. Interestingly, cells deficient in homologous recombination (HR) were hypersensitive to MNNG and arrested already in the first cell cycle after treatment. This implies that HR processes cytotoxic lesions induced by this methylating agents so as to allow the treated cells to pass through mitosis. However, this processing generates secondary lesions that kill the cells in the subsequent cell division.

Introduction

Chemotherapeutic methylating agents damage DNA either by methylation or DNA interstrand crosslink formation. Procarbazine, dacarbazine and temozolomide contain a single methyl group that can form DNA monoadducts at the N7 and O6 atoms of guanine, the N1 and N3 atoms of adenine and the N3 atom of cytosine. The majority of these adducts are repaired rapidly and efficiently by base excision repair, causing no cytotoxicity at doses used in the clinic. The only adduct shown to cause cell death is O-6-methylguanine (^{6me}G). The methyl group from the 6 position of guanine is usually removed by O6-methylguanine methyl transferase (MGMT), a suicide enzyme that is targeted to proteosomal degradation after transferring the methyl group to a cysteine residue inside its active site. Cells that overexpress MGMT are highly resistant to methylating agents, but its activity can be inhibited by a base analogue O6-benzylguanine that acts as a high affinity substrate for MGMT (Dolan et al., 1998).

Treatment of cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) brings about cell cycle arrest and cell death in mismatch repair (MMR) dependent manner. As hypothesized before, when replicative polymerase encounters ^{6me}G in the template strand, it attempts to incorporate a nucleotide that fits Watson-Crick base pairing the best. Due to steric reasons, this seems to be T although it was shown that depending on the polymerase used, it can be either T or C (Reha-Krantz et al., 1996; Singh et al., 1996). Both ^{6me}G-T and ^{6me}G-C are recognized by MutSα (Berardini et al., 2000; Duckett et al., 1996) and this is thought to initiate the so-called futile cycling of MMR-dependent excision and resynthesis by the polymerase. Finally, the polymerase is thought to re-initiate replication further downstream, leaving a ssDNA gap opposite the ^{6me}G. In the subsequent cell cycle, the ssDNA gap will be converted to a double strand break and cause cell cycle arrest.

The surprising thing about MNNG treatment is that the cell cycle arrest is activated only in the second S-phase after treatment (Stojic et al., 2004), although processing by MMR is expected to occur already in the first replication phase, when the ^{6me}G in the template strand mispairs with either T or C. Thus, the delayed mode of action of MNNG may be due to formation of secondary lesions that have to be essentially different in the first and the second S phase after treatment.

It has been reported previously that treatment of MMR-proficient cells with MNNG causes elevated levels of homologous recombination (Zhang et al., 2000). It could

therefore be assumed that homologous recombination is required for the processing of the secondary lesions, as was shown to be the case in yeast (Cejka et al., 2005).

Besides correcting mismatches that escape the proofreading activity of the polymerase during replication, MMR has been implicated also in the processing of recombination intermediates. In bacteria and yeast mismatches created during recombination are known to be addressed by MMR, which causes abortion of the heteroduplex formation thus acting antirecombinogenic (Surtees et al., 2004). The involvement of MMR proteins in mammalian recombination processes has been described for meiotic recombination and this only for MutL components of the MMR machinery (Baker et al., 1995; Baker et al., 1996; Edelmann et al., 1996). Possible role of MMR proteins in somatic recombination was demonstrated in $MSH2^{-/-}$ mouse ES cell line, which during gene targeting showed as efficient integration for nonisogenic construct as for isogenic (de Wind et al., 1995).

MMR-dependent processing of ^{6me}G adducts has been studied intensively. However, although the consequences of the ^{6me}G formation have been described in detail (Cejka et al., 2005; Kaina, 2004; Karran, 1995; Stojic et al., 2004), the molecular mechanisms of processing of this DNA modification has never been addressed successfully. In this work, I set out to identify the DNA metabolic pathways that are involved in the cytotoxicity of S_N1-type methylating agents such as MNNG in the living cells.

Results and Discussion

Uncoupling of DNA replication and MMR processing after MNNG treatment

Mismatch repair proteins have predominantly nuclear localization as can be expected by the role they play in the cell. MutSα is a heterodimer composed of MSH2 and MSH6 and is responsible for recognition of base mismatches and 1-2 nucleotide insertion-deletion loops. It was shown that MSH6 interacts with PCNA and is localized to replication foci (Kleczkowska et al., 2001). In order to follow the localization of MutSα depending on the cell cycle and treatment with MNNG, we synchronized HeLaMR cells (MGMT deficient, a kind gift of M. Bignami) with double thymidine block and released them in thymidine-free medium without (untreated) or with 0.2 μM MNNG. As shown in Figure 1A, in untreated cells foci of MutSα appeared in S and G2 phase of the cell cycle and followed the localization of PCNA. This is in accordance with previously published data (Kleczkowska et al., 2001) as MMR machinery is linked to replication in order to repair the mismatched base pairs that have escaped the proofreading activity of the polymerase. Also, it can be noticed that foci of MHS6 did not persist throughout the cell cycle but were prominent during the S and G2 phases and completely disappeared in G1. The pattern of MSH6 localization in cells treated with MNNG after the release from the double thymidine block was somewhat different. The number of foci in the first replication phase was higher than in untreated cells, but they were still colocalizing with PCNA, indicating that MSH6 is part of the replication machinery. After the first S phase, the difference between control and treated cells became obvious. The focal pattern of PCNA staining disappeared as could be expected, because the cells exit the S phase, but MSH6 continued to be localized in the discrete foci. This can be explained by the possibility that MutSα complex dissociates from PCNA and replication machinery upon mismatch recognition, and continues to stay bound to the mismatch. The same phenomenon was observed in *in vitro* system (Lau and Kolodner, 2003) where MutSα dissociated from PCNA upon binding of mismatched DNA.

In contrast to untreated cells, where foci of MSH6 disappeared after S-G2 phase together with those of PCNA, in treated cells MSH6 continued to be localized in very clear foci. Although cells continued to cycle as indicated by FACS analysis (Figure 1B) and by PCNA staining (big, centrally located foci, indicating late S phase) foci of MSH6 continued to persist independently of PCNA. Only in the very late time point, when the

cells were already arrested in G2 phase, did foci of MSH6 disappear. This indicates that MutSα was recruited to the sites of damage during two cell cycles, changing its localization already in the first S phase, but still residing on the damaged DNA in the second replication phase after MNNG treatment. As can be noted from Figure 1B, the progression through the first S phase in control synchronization (Figure 1Ba) and MNNG treated cells (1Bc) was identical, that is, the speed of replication fork was not influenced by the presence of ^{6me}G. This is contrary to the hypotesis of futile cycling, where the progression of the replicative polymerase would be temporarily blocked by the cycles of MMR provoked excision and resynthesis by the replicative polymerase. The speed of the S phase progression indicates that the polymerase itself is not stalled, but can freely continue across and downstream of the ^{6me}G residues in the template. MutSα following the replication fork can recognise the mispair, dissociate from PCNA and engage in attempts, uncoupled from the replication fork, to remove the mismatch.

MNNG treatment brings about MMR-dependent cell cycle arrest in the G_2 phase of the second cell cycle after treatment (Stojic et al., 2004). Presumably, methylation by MNNG gives rise to ^{6me}G, which in the subsequent replication phase pairs with T or C. The mismatch can then be addressed by MMR and activate the checkpoint. The specificity of MNNG treatment is its requirement for two replication cycles in order to arrest the cells. ^{6me}G residues are normally repaired by MGMT (methyl-guanine methyl transferase), an enzyme that catalyses the irreversible transfer of methyl group to an internal cysteine in the active site, thus targeting it for degradation. If MGMT reverts the damage before the replication fork encounters the ^{6me}G in the template strand, cells continue to replicate independently of their MMR status. Therefore, MGMT protects the cells from the cytotoxic effect of ^{6me}G. In many tumours, MGMT is not expressed due to hypermethylation of its promoter, rendering tumours more sensitive to methylating agents. In order to prevent MGMT activity during chemotherapeutic treatments with methylating agents, tumours can be pretreated with O-6-benzylguanine (BG), a nucleotide analogue that is a substrate for MGMT. Pre-treatment of cells with BG depletes MGMT activity, allowing for formation of stable ^{6me}G residues. Although the processing of the ^{6me}G-T mispair by MMR is expected to occur during the firs S phase, cells are allowed to pass the first mitoses, enter the second cell cycle and arrest only in the second G_2 phase post treatment. We wanted to examine if the ^{6me}G residue is required for MMR-dependent arrest only in the first replication phase after the treatment or in both S phases before the checkpoint arrest. We synchronized HeLa cells with 2

mM HU for 16 hours, and released them in HU-free medium. As indicated in the control synchronization, levels of MGMT protein do not vary during the cell cycle. When the cells were released in the medium containing 0.1 μM MNNG, level of MGMT was high enough to revert all ^{6me}G residues back into G. As shown by the levels of MGMT, this concentration of MNNG was not high enough to notably lower the amount on MGMT in the cells. As expected, cells did not arrest but continued to cycle. Contrary to this, when the cells were pretreated with O-6-benzylguanine (BG) for 16 hours, level of MGMT was notably reduced, corresponding to the description of MGMT as a suicide enzyme that is targeted for proteosomal degradation after the transfer of an alkyl group to the active-site cysteine. In this case, ^{6me}G residues were not reverted but enter replication where they were addressed by MMR. Consequently, cells arrested in G2/M phase of the second cell cycle after release/treatment as indicated at T36 and T60.

MGMT is a small protein of ~25kDa and after removal of O-6-benzylguanine from the medium is soon re-expressed to the normal levels. To assess if the presence of ^{6me}G is required in both the first and the second replication phase in order for the cell to arrest, we removed BG from the medium at T10 time point, when the cells have passed the first S phase but still did not enter the second cell cycle. The time since removal of BG (T10) to the point when the arrest becomes effective (T36) is sufficient to allow for the recovery of normal MGMT level (MGMT western blot, Figure 1B - panel 4.). Re-expression of MGMT allows for the removal of methyl group from guanidine and as a result, cells do not arrest but return to a normal cycling profile. From this, we can conclude that the presence of ^{6me}G is required in both cell cycles in order for cells to activate the checkpoint and arrest. On the other hand, if the BG is removed from the medium at T36, when the checkpoint has already been activated, cells continue to be arrested and don't recover from the arrest (Figure 1B, panel 5.). These results correspond to localisation pattern of MSH6 in Figure 1A, where MutSα is localised in discrete foci, presumably at the sites of damage, during two replication cycles. Foci disappear at the point when the arrest is effective (T36, second G2 phase after treatment) and when the reactivation of MGMT does not contribute to cell cycle progression.

Appearance of the cell cycle arrest this late after MNNG treatment suggests that the initial damage *per se* is not triggering the checkpoint activation. This long delay can only be explained by the requirement for processing of the initial damage during two cell cycles. We wanted to identify the repair pathway activated in MMR-dependent manner responsible for the generation of secondary lesions and delayed arrest.

The MRN complex is not required for the repair of MMR-induced intermediates after MNNG treatment.

In order to look for the pathway activated *in vivo* after MNNG treatment, we carried out a series of immunofluorescence studies, using a palette of antibodies that recognize proteins involved in different DNA metabolic transactions. Most of the DNA repair proteins have a tendency to translocate after damage into so-called DNA damage induced foci and colocalization of proteins in the same focus is indicative of their engagement in the same repair process. A candidate complex emerged after staining the cells with MLH1 and MRE11 antibodies. In untreated culture, a small proportion of cells showed foci of MLH1 and MRE11 and, interestingly, these foci were always colocalizing (Figure 2A). MRE11 is a component of the so-called MRN (MRE11-NBS1-RAD50) complex and is a key player in cellular response to DSBs (Assenmacher and Hopfner, 2004). The complex is an ATP-stimulated nuclease that degrades DNA in the 3'→5' direction and is involved in the resection of DNA ends during DSB repair, particularly by cleaving DNA hairpins formed on ssDNA. Involvement of the MRN complex in replication was shown by interaction of its NBS1 component with the E2F transcription factor near replication origins (Maser et al., 2001) and colocalisation of MRE11 with PCNA in replication foci (Mirzoeva and Petrini, 2003). The role of the MRN complex in prevention/repair of collapsed replication forks was shown in cell-free *Xenopus* egg extracts, where the absence of the complex resulted in many DSBs (Costanzo et al., 2001). Processing of ^{6me}G by the mismatch repair machinery could possibly leave a gap in the DNA that would be converted to a DSB in the following cell cycle, where the function of the MRN proteins in the stabilization of replication fork could play a key role. The possibility that MLH1 and MRE11 participate in the same pathway responsible for MNNG sensitivity was not confirmed, because the number of MLH1 or MRE11 foci did not increase after MNNG treatment (data not shown). As activation of the repair proteins is not always accompanied by their translocation into foci, we set out to check the phosphorylation status of NBS1 and MRE11 in 3 matched pairs of MMR-proficient and deficient cell lines. 293T Lα+ and Lα- is an isogenic cell pair in which the MMR status can be regulated with doxycyclin (Cejka et al., 2003). HCT116+chromosome 3 is a MMR proficient cell line derived from MMR deficient cancer cell line HCT116 by transfer of chromosome 3. HeLa clone 12 is derived from HeLa by selection with low doses of MNNG and is MMR deficient due to the lack of PMS2.

NBS1 is required for ATM autoactivation and phosphorylation (Lee and Paull, 2004) and is in turn phosphorylated by ATM on Ser343 (Lim et al., 2000). MRE11 protein is also phosphorylated *in vivo* (Dong et al., 1999), although the phosphorylation site is not yet known. As shown in Figure 2B, NBS1 is phosphotylated after MNNG treatment in all MMR proficient cell lines, while there is no NBS1 phosphorylation in MMR-deficient cells. The same is true for MRE11, whose hyperphosphorylated form is present in MMR proficient cells, but is absent from MMR deficient. The lack of the hyperphosphorylated form of MRE11 in 293TLα cells is linked to the presence of SV40 Large T-antigen that was reported to interfere with the MRN pathway (Digweed et al., 2002; Lanson et al., 2000; Wu et al., 2004).

Phosphorylation of NBS1 was apparent already at the 24 hour time point, when cells passed through one round of replication with ^{6me}G present in the DNA. Although the difference between MMR proficient and deficient cells becomes apparent at this time point, this is still not enough for the cells to arrest, indicating that the processing intermediates created in the first S-phase can be tolerated.

In order to assess if activation of the MRN complex is required for the repair of the lesions generated by MMR processing, we used two isogenic cell pairs defective in either NBS1 (NBS1 cells and NBS1 cells complemented with cDNA of NBS1 (Ito et al., 1999); a kind gift of K. Komatsu) or MRE11 (ATLD1 cells and ATLD1 complemented with cDNA of MRE11 (Carson et al., 2003); a kind gift of M. Weitzman). The MRN complex has been shown to play a crucial role in DNA damage signalling, mainly participating in the ATM pathway (Carson et al., 2003). Thus, one of the consequences of the loss of MRN function might be incorrect checkpoint activation in response to the damage. As we showed before (Stojic et al., 2004), the cell cycle profile of MMR proficient cells changes two cell cycles after MNNG treatment. NBS and NBS+NBS1 cells show the typical profile of MMR proficient cells with G2/M accumulation two cell cycles after treatment, at the 48 hours time point. Although NBS1 was phosphorylated on Ser343 in MMR dependent manner (figure 2B), it does not seem to be required for the checkpoint activation. The overall survival of the cells after MNNG treatment depends mostly on the repair efficiency and not on the signalling pathway activated in response to damage (di Pietro et al., 2003). In order to check the involvement of NBS1 in the repair of lesions created by MMR processing, clonogenic assays were preformed and, as shown in Figure 2D, there were no differences in survival between cells deficient and proficient in NBS1. Similar results were confirmed with ATLD-1 cells, where expression of

recombinant MRE11 did not influence cell cycle distribution (Figure 2E) or overall survival (Figure 2F) after MNNG treatment.

Interaction of MLH1 and MRE11 has been reported before (Her et al., 2002) and, although colocalization of MLH1 and MRE11 in untreated cells (Figure 2A) could indicate their involvement in a common pathway, it's clear from these results that this pathway is not necessary for initial checkpoint activation or for overall survival. These findings are consistent with the fact that the MMR-dependent cell cycle arrest does not require the ATM kinase (Stojic et al., 2004), which participates in the same pathway as the MRN complex. Also, the role of the MRN complex in the stabilization of replication forks is attributed to linking the free ends of a double strand break formed after the collapse of a replication fork (Assenmacher and Hopfner, 2004). In the case of MNNG treatment, we could not detect the presence of DSBs at any point after treatment, so the need for tethering free DNA ends by the MRN complex is presumably not required.

MMR processing of methylation damage activates homologous recombination.

MMR has been hypothesised to recognize ^{6me}G-T mispairs in the first round of replication after MNNG treatment and to attempt to correct them. As it's capable of removing the nucleotide incorporated in the newly-synthesized strand only, it will remove the T. This however does not repair the modified nucleotide in the template strand, namely ^{6me}G. The replicative polymerase will incorporate again a T opposite, that fits the best Watson-Crick pairing. According to this hypothesis, MMR and the polymerase will engage in futile cycles of incorporation and degradation of the newly-synthesised strand opposite of ^{6me}G, and eventually the polymerase will resume the replication downstream of ^{6me}G (for review, see (Bignami et al., 2000)). According to this model, there will be a gap created in the daughter strand whose filling-in will be blocked by ^{6me}G in the template strand. Gaps of this kind are highly susceptible to endonucleolytic cleavage in living cells, unless protected by ssDNA binding proteins (Wold, 1997). The presence of gaps in the DNA could be detected by the presence of damage-induced foci of ssDNA-binding proteins. One of those proteins tested was RAD51, a homologue of bacterial RecA, also known as a recombinase, because of its role in the initial steps of homologous recombination. Its localization to the sites of damage is dependent on BRCA2 with which it interacts through BRC domains (Pellegrini et al., 2002). RAD51 displaces RPA from the ssDNA and promotes strand

invasion and formation of D-loop in the intact homologous dsDNA (Shin et al., 2004). In 293TLα cells, foci of RAD51 became apparent in MMR-proficient cells already at the 24 hour time point, but peaked 48 hours after MNNG treatment (Figure 3A). The highest number of foci corresponded to the time point when the G2 arrest of MMR-proficient cells was apparent. There were no foci of RAD51 in MMR-deficient cells at any time point after treatment indicating that the damage created by MNNG was converted to recombination intermediates in MMR-proficient cells only. As we noted before, RPA, another ssDNA binding protein, forms MNNG induced foci specifically in MMR-dependent manner (Stojic et al., 2004). They were shown to colocalize with γ-H2AX foci, indicating that they form at the sites of chromatin remodelling in damaged DNA. We tested if the RAD51 foci representing sites of ongoing recombination can also be localized to the sites of RPA-γH2AX localization. For technical reasons, we used MMR-proficient HeLa cells and stained them with both RAD51 and RPA antibodies. As shown in Figure 3B, 48 hours after MNNG treatment the number of both RAD51 and RPA foci peaked, with majority of the foci colocalizing as indicated by the yellow colour of the merged images. Colocalization of RAD51 and RPA at the sites of single-stranded DNA regions has been reported previously and is indicative of their common involvement in recombination repair (Raderschall et al., 1999).

From this result, we observed that homologous recombination pathway is activated in a MMR-dependent manner. *In vivo* consequence of repair by homologous recombination can be observed at cytological level as sister chromatid exchanges (SCE). They are the consequence of homologous recombination associated with stalled or collapsed replication forks, while true DSBs in mammalian cells are almost exclusively repaired by non-cross-over mechanism (sister chromatid gene conversion) thus not rendering cytological phenomenon of SCE (Johnson and Jasin, 2000; Richardson et al., 1998).

It has been reported previously that MNNG treatment brings about elevated levels of SCE and their formation has been specifically attributed to ^{6me}G (Kaina, 2004; Kaina and Aurich, 1985). Differential sensitivity of MMR-proficient and -deficient cells to MNNG treatment is dependent on the presence of ^{6me}G, as cells expressing high levels of MGMT are highly resistant to killing by this agent, irrespective of their MMR status (Kaina et al., 1997). In order to test the dependence of SCE formation on MMR status, we analyzed both MMR-proficient and -deficient 293TLα cells after MNNG treatment. In order to distinguish sister chromatids in mitotic chromosomes, the cells have to be grown for two cell cycles in the presence of BrdU. Chromosomes of such cells can be distinguished,

because one sister chromatid will have both DNA strands labelled with BrdU, while the other sister chromatid will have BrdU incorporated in only one strand of DNA. As MMR proficient cells needed two replication cycles to activate the checkpint and arrest, we assessed the SCE fomation in the presence of MNNG after 48 hours (MNNG added together with BrdU) but also after 24 hours, when the cells have passed only one replication cycle in the presence of ^{6me}G (MNNG added 24 hours after the addition of BrdU). As shown in Figures 3C and 3D, elevated levels of SCE can be observed in 293TLα+ cells only 48 hours after MNNG treatment. Number of SCE per cell rose from average 3.14 to about 4 times higher average level with many cells having around 20 SCEs. This correlates with previous findings, where it was shown that ^{6me}G needs additional processing to be converted into a SCE-inducing lesion (Karran et al., 1993) and clearly MMR is a crucial component of the process.

At this point it has to be noted that SCE are outcomes of recombination-dependent processing going on during replication. However, it cannot be concluded that SCE are the deleterious structures that finally arrest the cells. Two cell cycles after treatment, a large proportion of MMR-proficient cells are arrested in the G2 phase of the cell cycle but in order to score SCEs, cells have to pass to mitosis. Therefore, only the cells that escaped the arrest can be scored, while those arrested never pass the G2/M boundary and their chromosomes cannot be visualized. We can thus assume that the process giving rise to SCEs is stalled in the majority of the cells and not efficient enough to repair the intermediates and allow the cells to recover from the G2 arrest. Only a small proportion of the cells deal with the damage successfully and, as a consequence of this repair process, SCEs are visible in their mitotic chromosomes.

Homologous recombination is required for initial bypass and overall survival after formation of ^{6me}G

Experiments in which relocalization of RAD51 and RPA proteins was observed in MMR-proficient cells and formation of elevated levels of SCEs depended on MMR status indicated that homologous recombination pathway is indeed activated in MMR-dependent manner. Secondary lesions created by MMR processing of ^{6me}G seem to be addressed by homology-dependent repair. In order to test the response of MMR-proficient cells with regard to their ability to carry out recombination repair, we used homologous recombination impaired cell lines lacking the RAD51 paralogs XRCC2 and

XRCC3. These proteins are members of the RAD51 family (composed of RAD51B, RAD51C, RAD51C, XRCC2 and XRCC3) and initial findings suggested that they are required for the early steps of homologous recombination. Their ability to repair DSBs is almost completely abolished (Johnson et al., 1999; Pierce et al., 1999) and mouse knockouts are embryonicly lethal (Deans et al., 2000). Cells lacking XRCC2 or XRCC3 were shown to be unable to form damage-induced RAD51 foci (Bishop et al., 1998; O'Regan et al., 2001) suggesting the requirement for these polypeptides in formation of RAD51-ssDNA filaments. Recent results indicate involvement of RAD51 paralogs in later steps of homologous recombination. Five members of the family form two distinct complexes in living cells: one is composed of RAD51C-XRCC3 and the other comprises RAD51B-RAD51C-RAD51D-XRCC2 (Liu et al., 2002; Masson et al., 2001). The RAD51C-XRCC3 has been recently identified as a component of the mammalian resolvase complex (Liu et al., 2004).

Irs1 cells are mutated in *XRCC2* gene; they fail to form RAD51 foci in response to DNA damage and are hypersensitive to a variety of DNA damaging agents such as the crosslinking agent Mitomycin C (MMC) (Jones et al., 1987). Introduction of cDNA of human *XRCC2* restored DNA damage resistance and RAD51 focus formation (O'Regan et al., 2001). Irs1SF cells are defective in XRCC3 and, like Irs1, are hypersensitive to DNA damaging agents. AA8 is the parental cell line, able to carry out homologous recombination.

In order to assess the influence of functional recombinational repair on survival of MNNG-treated cells, we treated two pairs of cell lines: Irs1 and Irs1 complemented with cDNA of XRCC2; and Irs1SF and its parental, homologous recombination proficient cell line AA8. As indicated in Figure 4A, cell survival after MNNG treatment was highly dependent on functional recombination repair. Irs1 and Irs1SF were substantially more sensitive to MNNG than their recombination-proficient counterparts. Extreme sensitivity of Irs1SF cells might be due to the role of XRCC3 in the resolution of Holliday junctions, where in the absence of efficient resolution, recombination intermediates signal and eventually arrest the cell cycle. In both cases, it's clear that functional recombinational repair is rescuing some of the intermediates arising after MNNG treatment.

As the lack of recombination efficiency renders cells more sensitive to MNNG treatment, we also wanted to know if this influences the cell cycle distribution and arrest after treatment. Therefore, we treated the cells and analyzed their DNA content at time

points correlating to the length of their cell cycle (approximately 16-18 hours). Figure 4B shows that recombination proficient cells, Irs1/XRCC2 and AA8, showed a typical cell cycle profile of MMR-proficient cells after MNNG treatment. They required two DNA replication cycles to activate the checkpoint and arrested in G2/M phase. Contrary to this, the recombination impaired cell lines Irs1 and Irs1SF show accumulation of G2/M phase cells already 16 hours after MNNG treatment, during which time the cells were able to replicate their DNA only once. The fact that both XRCC2 and XRCC3 deficient cell lines behaved the same shows that bypass of the lesions generated after MNNG treatment required recombination as a whole rather than merely some of its individual components.

In order to follow the cell cycle of MMR-dependent arrest in recombination proficient and -deficient cells in detail, we synchronized the Irs1 and Irs1/XRCC2 cells in G1/S phase of the cell cycle and released them in MNNG-containing medium. This pair of cells was chosen over Irs1SF and AA8 because they are nearly isogenic (stabile transfection of XRCC2 cDNA).

The cells were synchronized at the G1/S transition with 2 mM hydroxy-urea for 16 hours (T0) and released in HU-free medium containing MNNG. Control synchronization showed that both HR proficient and deficient cells recovered completely from the HU-induced arrest. The cells were then collected at the indicated time points and processed for cell cycle analysis. Progression of the Irs1/XRCC2 cells followed an already known pattern of MMR proficient cells, where they clearly re-entered the second cell cycle after treatment as indicated at T10, T12 and T16 time points by an increase in the G1 fraction. Contrary to that, the Irs1 cells were still in G2 phase of the cell cycle at these time points, with only a very small proportion of the cells re-entering the second cycle. The difference was also obvious at the later time point where many more Irs1 cells can be detected in the sub-G1 region of the histogram, indicating a larger proportion of dead cells.

A delayed cell cycle arrest, namely the requirement for two replication cycles after MNNG treatment in MMR proficient cells, has been a phenomenon observed long time ago, but so far there was no mechanistic model given to satisfy the observed results. MMR machinery is expected to recognize ^{6me}G-T mispairs already during the first replication after MNNG treatment. Unless repaired or bypassed, this processing is expected to be recognized by checkpoint proteins that will activate the cell cycle arrest. From the cell cycle analysis of recombination deficient cells, we show that during the

first cell cycle, MMR indeed recognizes the damage (presumably ^{6me}G) and creates secondary lesions. In the absence of homologous recombination, these lesions cannot be bypassed and therefore HR-deficient cells arrest already in the first G2 phase after treatment. In the presence of HR, secondary lesions can be bypassed during the first S/G2 phase, but apparently the DNA still contains structures that will become deleterious in the subsequent replication.

The simplest model that can be drawn from these results is presented in Figure 5A. In this model, MMR processing of the ^{6me}G-containing template strand leaves a gap in the newly-synthesised strand, because any nucleotide incorporated opposite ^{6me}G will be recognized as incorrect and will be removed by the exonuclease component of the mismatch repairosome. The role of RAD51 paralogs, XRCC2 and XRCC3 might be to bind to the gap and protect it during the subsequent phases of cell division. XRCC2 and XRCC3 bind ssDNA and it was reported that two complexes of RAD51 paralogs bind DNA gaps and nicks, indicating that gaps *per se* can be recombinogenic, without the need for free 3' end (Liu et al., 2002). According to this model, RAD51 paralogs would prevent access of RPA to the single-stranded fragments; therefore gaps would remain "invisible" to the checkpoint machinery, as ssDNA is the substrate for the DNA damage sensors only if coated with RPA (Zou and Elledge, 2003). In the subsequent cell cycle, the replication fork would encounter the gapped fragment that would result in the collapse of the replication fork. In this case, the secondary lesion would be created already in the first S-phase but would be covered by ssDNA binding proteins (XRCC2, XRCC3) that would protect it till the next replication phase.

Another possibility is that progression through the first S phase is dependent on recombination bypass, something similar to so-called template switching (Figure 5B). Polymerase stalling opposite the ^{6me}G would direct the invasion of the 3' end of the newly-synthesised strand to the newly-synthesised strand of the sister chromatid. After elongation and formation of the second holiday junction, resolution would occur in gene-conversion manner, without cross-over. As shown in Figure 3C, there were no sister chromatid exchanges in 293TLα+ cells after the first cell cycle. This is consistent with the fact that resolution of double Holliday junctions in mammalian cells results in non-cross-over mechanism (Helleday, 2003). Resolution gives rise to nicks in the newly-synthesised strand, which can be sealed, but it's possible that these nicks serve as strand discrimination signal for MMR initiation that will direct MutSα to recognize ^{6me}G-C pair and again remove the C from the daughter strand. ^{6me}G-C pairs are recognized by MutSα

with a similar efficiency as ^{6me}G-T mispairs (Duckett et al., 1996) and are repaired efficiently in *in vitro* systems (Franziska Fisher, unpublished data). As the polymerase has already moved downstream from the lesion, a gap in the daughter strand is created and cannot be refilled due to the persistence of ^{6me}G in the template strand. The consequence in the second cell cycle is again a collapse of the replication fork. In both models, a collapsed replication fork can induce break-induced replication (BIR), where the 3' end invades the duplex DNA upstream of ^{6me}G and the polymerase tries to extend the invading strand. Again, it will encounter ^{6me}G in the template strand and any nucleotide incorporated opposite (T or C) will be recognized by MMR, but, in this case, there will be no homologous DNA duplex that can serve as template. At this point, the replication fork would be blocked and signal strongly to the checkpoint machinery (Figure 5, Major product). The collapse of the replication fork in MMR-proficient cells can explain the elevated levels of SCE two cell cycles after MNNG treatment. Recombination intermediates in break-induced replication are resolved in non-cross over manner, joining the template DNA and the newly synthesised DNA at the point of the collapse. It has to be noted that a small proportion of cells manage to elongate the daughter strand across the ^{6me}G. These are most likely cells that enter mitosis and can be scored for SCEs. On the other hand, a majority of the cells cannot elongate the invading strand and arrest in the G2 phase, with possible S phase arrest in p53 proficient cells (Stojic et al., 2004). Also, this is most likely the moment where point mutations occur. If the polymerase manages to elongate the invading strand, it is possible that it will incorporate a T, which will cause GC to AT transitions (Figure 5, Minor product).

The results shown in Figure 1, where the removal of ^{6me}G in the second cell cycle prevented the arrest can be explained now. Activation of MGMT allows for removal of the methyl group, thus leaving guanine in the template strand. The gap that was persistent can now be filled-in before the replication fork in the second S phase encounters it. Even if the replication fork progressed past the gap, MGMT could remove the methyl group in the template strand and the polymerase could synthesise past it. This would leave an unmodified substrate for the repair of the collapsed fork, where the free end could invade the sister chromatid and restart the fork.

The models presented here need additional supporting experiments, but this is the first time that distinction between the first and the second replication in the presence of MNNG could be made.

Materials and Methods

Cell lines

The 293T Lα cell line was established in our laboratory and was propagated as described (Cejka et al., 2003). HeLa mex$^-$ and MRC5 cells were maintained in DMEM (OmniLab) supplemented with 10% fetal calf serum (FCS, Life Technologies), penicillin (100 U/ml) and streptomycin (100 μg/ml). HeLa mex$^-$ cells were a kind gift of Margherita Bignami. The hMLH1-deficient human colon cancer cell line HCT116 and its MMR-proficient subline HCT116 + ch3 were maintained in McCoy's 5A medium (OmniLab) with 10% FCS. The chromosome-complemented cell line was maintained in medium containing 400 μg/ml G418. Homologous recombination-impaired Chinese hamster cell line Irs1 is mutated in XRCC2 and the Irs1/XRCC2 line was complemented with human cDNA of XRCC2. Both cell lines were provided by John Thacker. XRCC3 deficient cells and their proficient parental cell line AA8 were a gift from Orlando Schaerer's group. All four Chinese hamster cell lines were maintained in DMEM:F10/1:1 (OmniLab), 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml). NBS1 cells and those complemented with NBS1 cDNA were produced in the lab of Dr. Kenshi Komatsu and provided by Dr. Yosef Shiloh. They were maintained in DMEM supplemented with 10% FCS and the complemented cell line with additional 100 μg/ml Hygromycin B. For clonogenic assays, the medium was supplemented with 20% FCS. ATLD1-vector and ATLD1-MRE11 cells were a kind gift of Dr. Matthew Weitzman and were grown in DMEM, 20% FCS with 0.1 μg/ml puromycin. To inhibit MGMT activity, HCT116, HCT116+ch3, MRC5, NBS1 and ATLD1 cells were pre-treated with 10 μM O^6-benzylguanine two hours prior to MNNG treatment. All cell lines were cultured at 37°C in a 5% CO_2 humidified atmosphere.

Chemicals and irradiations

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG, Sigma) was dissolved in DMSO and stored as 1 M solution at -20°C in the dark. O^6-benzylguanine (Sigma) was dissolved in ethanol and stored at -80 °C. Hydroxyurea (HU, Sigma) and doxycycline (Dox, Clontech) were dissolved in water and stored at -20°C.

Cell synchronization

HeLa (Figure 1) and Irs1 and Irs1/XRCC2 (Figure 4) cells were grown to 30-40% confluency and 2 mM HU was added for 16 hours. The cells were washed twice with PBS and incubated in fresh medium containing 0.2 or 2 μM MNNG. HeLa cells were incubated in the presence of benzyl-guanine as described in the text. The cells were harvested at the indicated time points and processed for cell cycle analysis.

Cell Cycle analysis

The cells were trypsinised, washed once in PBS and resuspended in 70% ethanol. On the day of the analysis, the cells were washed again in PBS, incubated for half an hour with RNase A (100 μg/ml, Sigma) at 37°C and stained with propidium iodide (20 μg/ml, Sigma). Analysis was done using Beckman Coulter FC500 cytometer.

Antibodies and immunoblotting

Anti-MLH1 (554072) and anti-PMS2 (556415) monoclonal antibodies were from BD Pharmingen, anti-β-tubulin (D-10), anti-TFIIH p89 (S-19), anti-PCNA (PC10) and anti BRCA1 (D-9) from Santa Cruz Biotechnology, anti-MRE11 (Ab-1) and anti-RPA p34 (Ab-3) from Oncogene, anti-NBS1 from Novus Bilogicals, anti-phospho-NBS1 (Ser-343) from Cell Signaling, rabbit polyclonal FBE2 anti-RAD51 was a kind gift of Stephen West. The polyclonal rabbit anti-MSH6 antibody was produced and purified in our laboratory. Immunoblotting and total protein extractions were performed as described previously (Cejka et al., 2003)

Immunofluorescence studies.

Cells grown on glass cover slips were treated or mock-treated with MNNG and incubated for the indicated time periods. Fixation was done in 3.7% formaldehyde/PBS (15 min, 4°C) followed by permeabilization in 0.2% Triton X-100/PBS (5 min, 4°C). In the case of anti-PCNA, the cells were fixed in ice-cold methanol (20 min, -20°C). The coverslips were blocked with 3% Low Fat Milk/PBS and incubated with anti-PCNA, anti-MSH6, anti-MRE11, anti-MLH1, anti-RAD51, or anti-RPA p34 antibodies. After washing, the cells were incubated with FITC–conjugated anti-rabbit or anti-goat antibodies (Sigma), and TR-conjugated anti-mouse antibodies (Abcam) for 1 h at 37°C. The nuclei were counter-stained with DAPI (0.1μg/ml, Sigma). Images were captured on a Leica DC 200 fluorescence microscope fitted with a CCD camera.

Sister Chromatid Exchange (SCE) assay

To differentially label sister chromatids, the cells were incubated for 48 hours with 10μM BrdU (two cell cycles). Assessing the effect of MNNG was accomplished by the addition of 0.2 μM MNNG 24 or 48 hours before harvesting. To increase the number of mitotic cells, 0.1 μg/ml colcemid was added for the last 2 hours. The cells were trypsinized, washed once in PBS and resuspended by gentle vortexing in 3 ml of 75mM KCl, followed by 10 minutes incubation at 37°C to allow cell swelling. The cells were pelleted and resuspended in freshly prepared Carnoy's fixative (Methanol:Acetic acid/3:1). After 30 minutes incubation, the cells were spun down and resuspended again in sufficient amount of fixative to achieve the desired cell concentration. The cell suspension was dropped on ice-cold, wet glass slides and allowed to air dry. After drying, the slides were incubated for 15 min in 10 μM aqueous solution of Hoechst 33258 and rinsed twice in water only. To differentiate sister chromatids, slides were immersed in 2xSSC solution and exposed to strong light for 45 minutes. After washing, the slides were stained in 3.5% giemsa solution in Soerensen buffer, pH 6.8 for 10 minutes and rinsed 5-6 times in tap water. Number of SCE events was counted in 50 metaphases and represented in Fig.3D.

Clonogenic survival assay

The cells were treated with the indicated concentrations of MNNG for 2 hours, trypsinized and counted. Serial dilutions of control and treated cells were plated and left for 14-20 days, after which colonies were stained with 10% giemsa and counted.

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Figure legends

Figure 1.

Mismatch repair is required during two replication cycles for cells to arrest after MNNG treatment. (A) Nuclear localization of PCNA and MutSα throughout the cell cycle of MNNG-treated and untreated cells. MMR-proficient HeLaMR cells were synchronized with double thymidine block, released and treated, and processed for indirect immunofluorescence staining as described in Materials and Methods. (B) The presence of 6-methylguanine, which is addressed by mismatch repair, is required in both first and second replication cycles after treatment. HeLa cells were synchronized in the G_1/S phase of the cell cycle with 2 mM hydroxy-urea for 16 hours with (Bc, Bd and Be) or without (Ba, Bb) 10 μM O-6-benzylguanine (BG). At the point of release, the cells were treated with 0.1 μM MNNG and fresh BG was added (Bc, Bd and Be). The cells arrested only when BG was present during the two cell cycles after treatment (Bc) or if it was removed at T36 (Be) when the checkpoint was already activated. If BG was removed at T10 (Bd) and MGMT was resynthesized before the cells enter the second S phase, there was no arrest and at T60 the cells had a typical asynchronous profile.

Figure 2.

The MNR complex is activated but not required for MMR-dependent processing of MNNG damage. (A) Indirect immunostaining for MLH1 and MRE11 showing colocalization of foci in untreated MRC5 cells. Only small percentage of cells was showing such foci, but they were colocalizing in each case. (B) NBS1 was activated upon MNNG treatment in MMR proficient cells already 24 hours after treatment. Activation of NBS1 was assessed by antibody against phosphorylated Ser343. Hyperphosphorylated form of MRE11 was present in HeLa and HCT116 + chr3 at later time points, represented by the slower-migrating form of the protein. (C) Cell cycle analysis of a matched pair of cell lines, one deficient in NBS1, the other complemented with NBS1 cDNA. The figure shows that the presence or absence of NBS1 does not influence G2 arrest after treatment with 0.2 μM MNNG. (D) Clonogenic assay of the same pair of cell lines, showing that survival after MNNG treatment is not influenced by the NBS1 protein. (E) FACS analysis of MRE11 mutated ATLD1 cells and a matching line complemented with MRE11cDNA after MNNG treatment. Both cell lines showed a

similar pattern of arrest, which was not dependent on the presence of functional MRE11. (F) Clonogenic assay of the same pair of cell lines showing that survival after MNNG treatment is not influenced by the MRE11 protein.

Figure 3.

Mismatch repair dependent processing of methylating damage activates homologous recombination. (A) Formation of RAD51 foci in 293T Lα cells upon treatment with 0.2 μM MNNG. Foci started to appear at the 24 hour time point only in MMR-proficient cells and their number peaked 48 hours after treatment. (B) Co-immunostaining of MMR-proficient HeLa cells showing colocalisation of RPA and RAD51 foci after 0.2 μM MNNG treatment. (C) Representative images of metaphase spreads of 293T La+ cells stained to differentiate sister chromatids of individual chromosomes. Only MMR-proficient cells showed elevated levels of sister chromatid exchanges 48 hours after MNNG treatment. (D) Quantification of sister chromatid exchange (SCE) events in 293T Lα cells after 0.2 μM MNNG treatment showing elevated SCE level in MMR-proficient cells.

Figure 4.

Homologous recombination is required for cell survival after MNNG treatment (A) Colony survival assay of homologous recombination impaired cell lines after MNNG treatment. Cell lines deficient in the RAD51 paralogs XRCC2 (Irs1) and XRCC3 (Irs1SF) are hypersensitive to MNNG compared to their recombination-proficient counterparts. (B) Cell cycle analysis of cell lines deficient (Irs1 and Irs1SF) and proficient (Irs1/XRCC2 and AA8) in homologous recombination after 2 μM MNNG treatment. Cells that can carry out recombination repair arrest two cell cycles after the treatment, while those defective in such repair start arresting much sooner and show increased amount of apoptotic cells correlating with poor survival capability. Cell cycle of these cells is approximately 16-18 hours. (C) Homologous recombination is required to bypass the first cell cycle after MNNG treatment. Isogenic cell lines (Irs1 and Irs1/XRCC2) were synchronized at the G1/S transition with 2 mM hydroxy-urea and treated with 2 μM MNNG. The cells were collected at the indicated time points and their DNA content was analyzed. Homologous recombination-deficient cells (Irs1) show arrest in the first

G2 phase after treatment, while the proficient cells (Irs1/XRCC2) went through the first cell cycle only to arrest in the second G2 phase after treatment.

Figure 5.

A possible model for the involvement of homologous recombination repair in MMR-mediated cytotoxicity of O-6-methylguanine.

MODEL A. The MMR processing of the ^{6me}G/T or ^{6me}G/C mismatch leaves a gap in the newly-synthesised strand. The gap is bound by RAD51 paralogues, thus preventing the binding of RPA and activation of the checkpoint. In the subsequent cell cycle, replication fork encounters the gap left over from the previous cycle and the fork collapses.

MODEL B. Stalling of the filling-in polymerase or rounds of excision and incorporation direct the 3' end of the newly synthesised strand to invade the sister chromatid and by-pass the ^{6me}G. The replicative polymerase can continue downstream of the lesion. Double Holliday junction will be resolved in a non-cross-over manner, but the nicks resulting from the resolvase cleavage can direct the MMR to excise the C opposite ^{6me}G. As the gap cannot be filled because of the persistence of ^{6me}G in the template strand, it will be converted into a collapsed replication fork in the next replication phase.

The collapsed replication fork can then be repaired by break-induced replication (BIR) but only if the gap in the template chromatid can be filled. As the ^{6me}G persists, neither the replicative polymerase, nor the polymerase engaged in the extension of the invading strand can bypass the ^{6me}G. Unlike in the first cell cycle, at this moment there is no sister chromatid that can be used as non-damaged template. Small persentage of cells that do manage to repair such break are very likely to acquire GC to AT transitions because incorporation of T opposite of ^{6me}G is less likely to elicit MMR-dependent excision, thus allowing for filling-in the gap and providing the substrate for the invading 3' end of the broken chromatid.

Figure 1

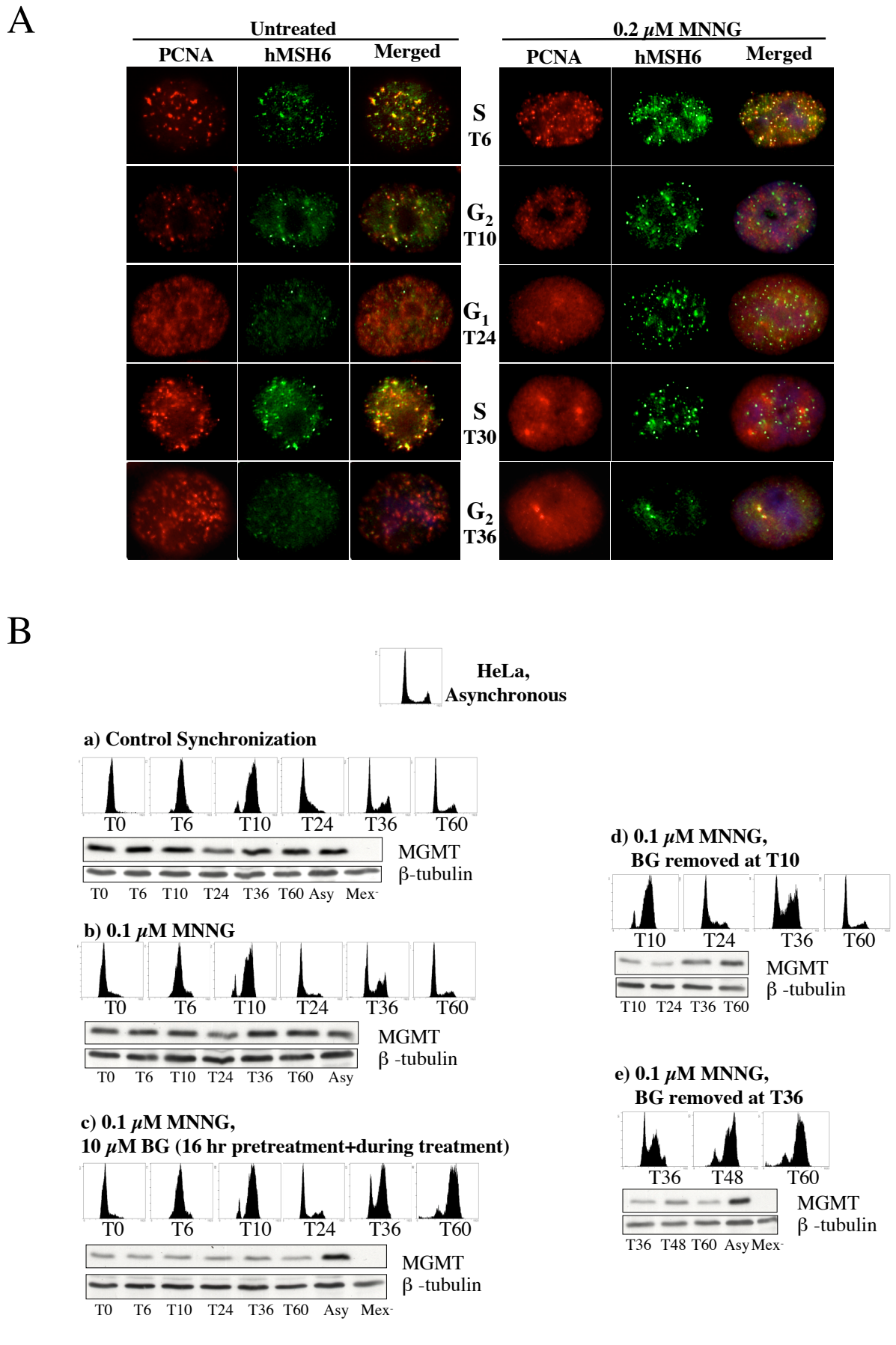

A
Untreated
PCNA
hMSH6
Merged
0.2 μM MNNG
PCNA
hMSH6
Merged
S
T6
G2
T10
G1
T24
S
T30
G2
T36
B
HeLa,
Asynchronous
a) Control Synchronization
T0 T6 T10 T24 T36 T60
MGMT
β-tubulin
T0 T6 T10 T24 T36 T60 Asy Mex-
b) 0.1 μM MNNG
T0 T6 T10 T24 T36 T60
MGMT
β -tubulin
T0 T6 T10 T24 T36 T60 Asy
c) 0.1 μM MNNG,
10 μM BG (16 hr pretreatment+during treatment)
T0 T6 T10 T24 T36 T60
MGMT
β -tubulin
T0 T6 T10 T24 T36 T60 Asy Mex-
d) 0.1 μM MNNG,
BG removed at T10
T10 T24 T36 T60
MGMT
β -tubulin
T10 T24 T36 T60
e) 0.1 μM MNNG,
BG removed at T36
T36 T48 T60
MGMT
β -tubulin
T36 T48 T60 Asy Mex-

Figure 2

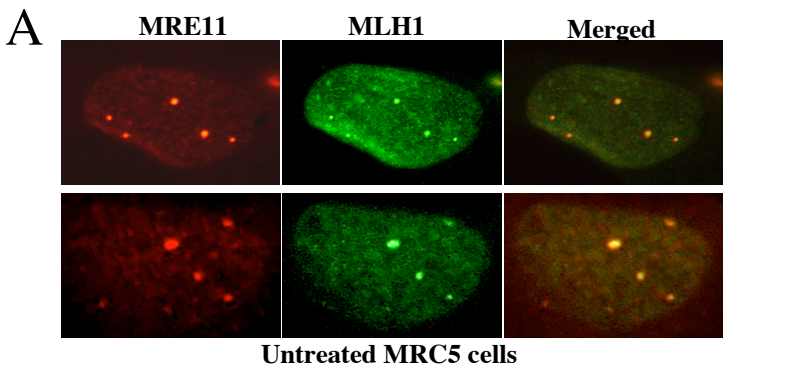

A
MRE11
MLH1
Merged
Untreated MRC5 cells
B
C
0.2 μM MNNG
24 48 72
Phospho-NBS1
NBS1
MRE11
Lα+
Lα-
HCT116 + Chr3
HCT116
HeLa
HeLa-clone 12

Figure 2

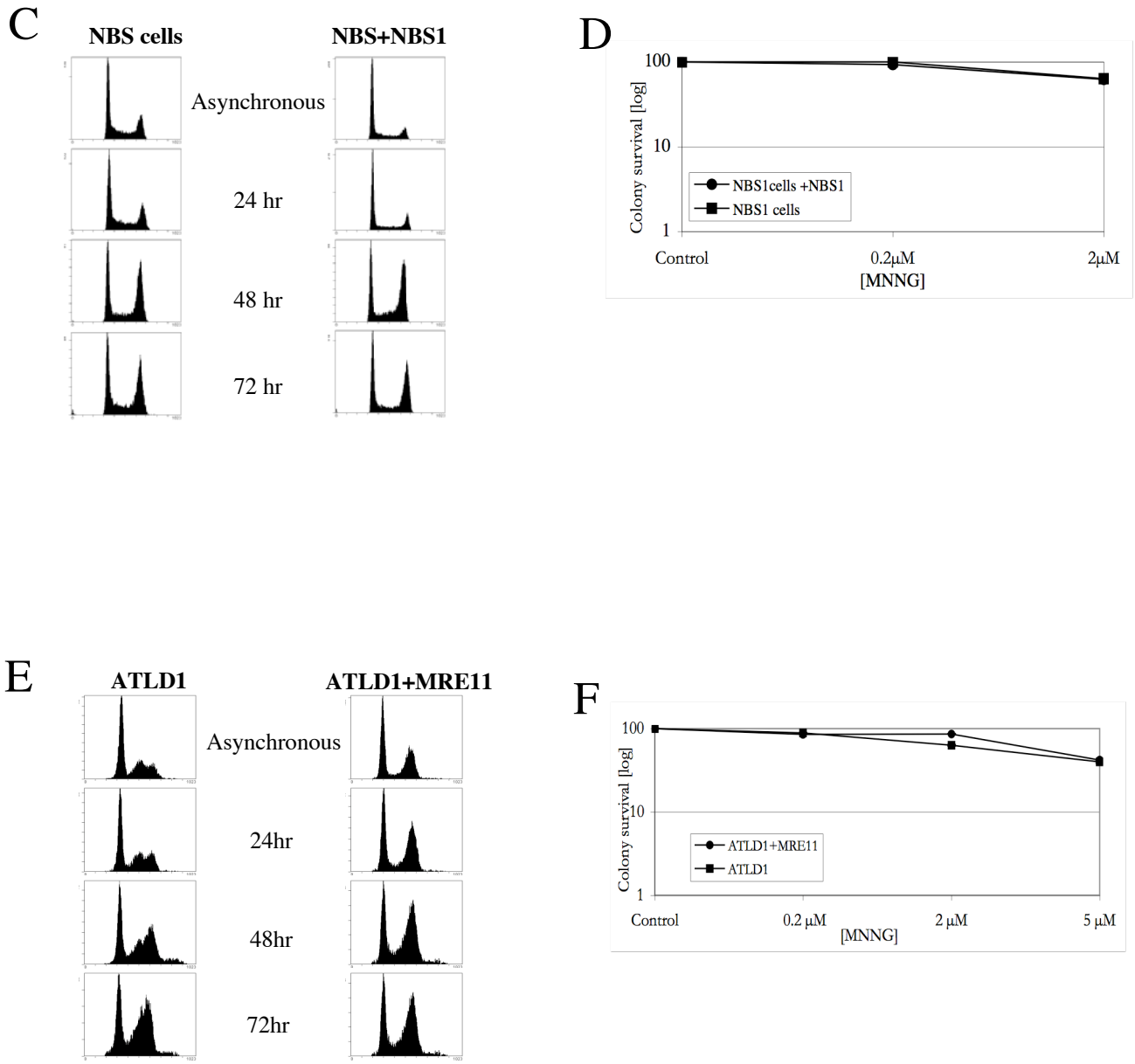

C
NBS cells
NBS+NBS1
Asynchronous
24 hr
48 hr
72 hr
D
100
10
1
Colony survival [log]
NBS1cells +NBS1
NBS1 cells
Control
0.2µM
2µM
[MNNG]
E
ATLD1
ATLD1+MRE11
Asynchronous
24hr
48hr
72hr
F
100
10
1
Colony survival [log]
ATLD1+MRE11
ATLD1
Control
0.2 µM
2 µM
5 µM
[MNNG]

Figure 3

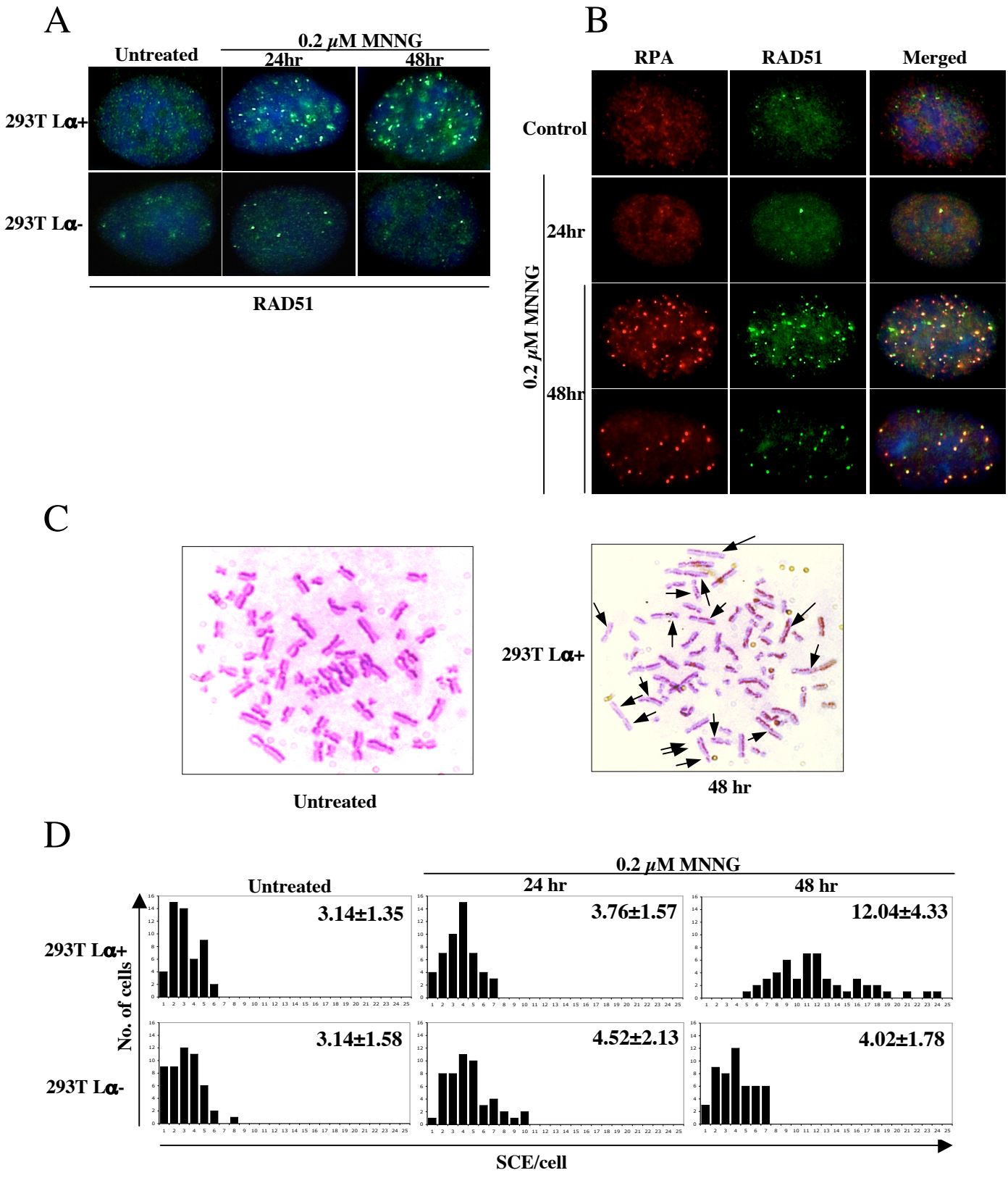
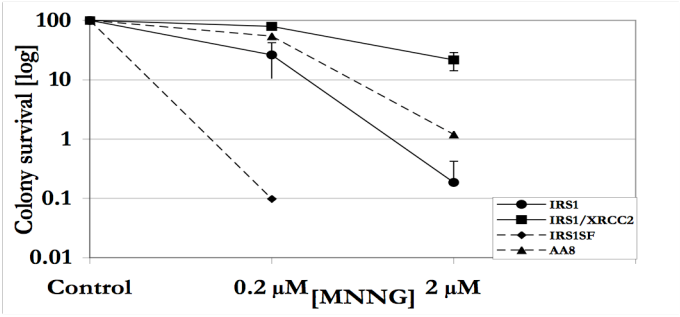

A
Untreated
0.2 μM MNNG
24hr
48hr
293T Lα+
293T Lα-
RAD51
B
RPA
RAD51
Merged
Control
0.2 μM MNNG
24hr
48hr
C
293T Lα+
Untreated
48 hr
D
0.2 μM MNNG
Untreated
24 hr
48 hr
293T Lα+
293T Lα-
No. of cells
3.14±1.35
3.76±1.57
12.04±4.33
3.14±1.58
4.52±2.13
4.02±1.78
SCE/cell

Figure 4

A


100
10
1
0.1
0.01
Colony survival [log]
Control
0.2 µM
[MNNG]
2 µM
IRS1
IRS1/XRCC2
IRS1SF
AA8

B

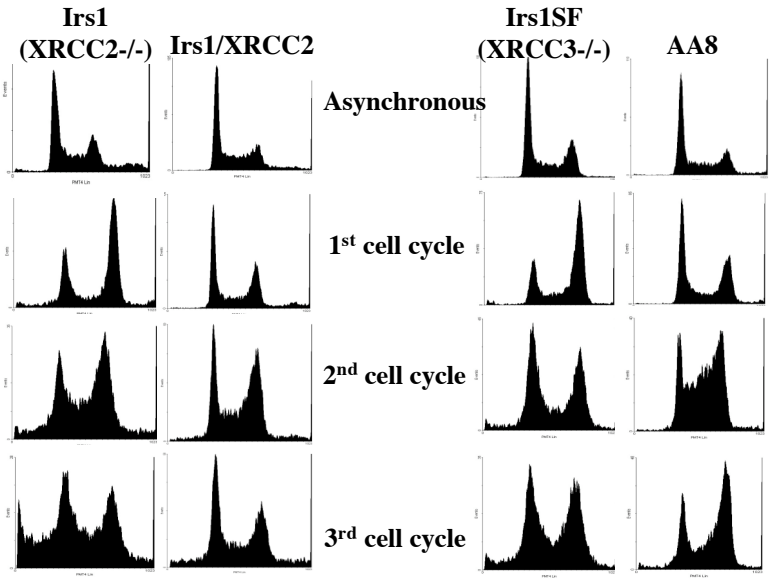

Irs1
(XRCC2-/-)
Irs1/XRCC2
Irs1SF
(XRCC3-/-)
AA8
Asynchronous
1st cell cycle
2nd cell cycle
3rd cell cycle

Figure 4

C

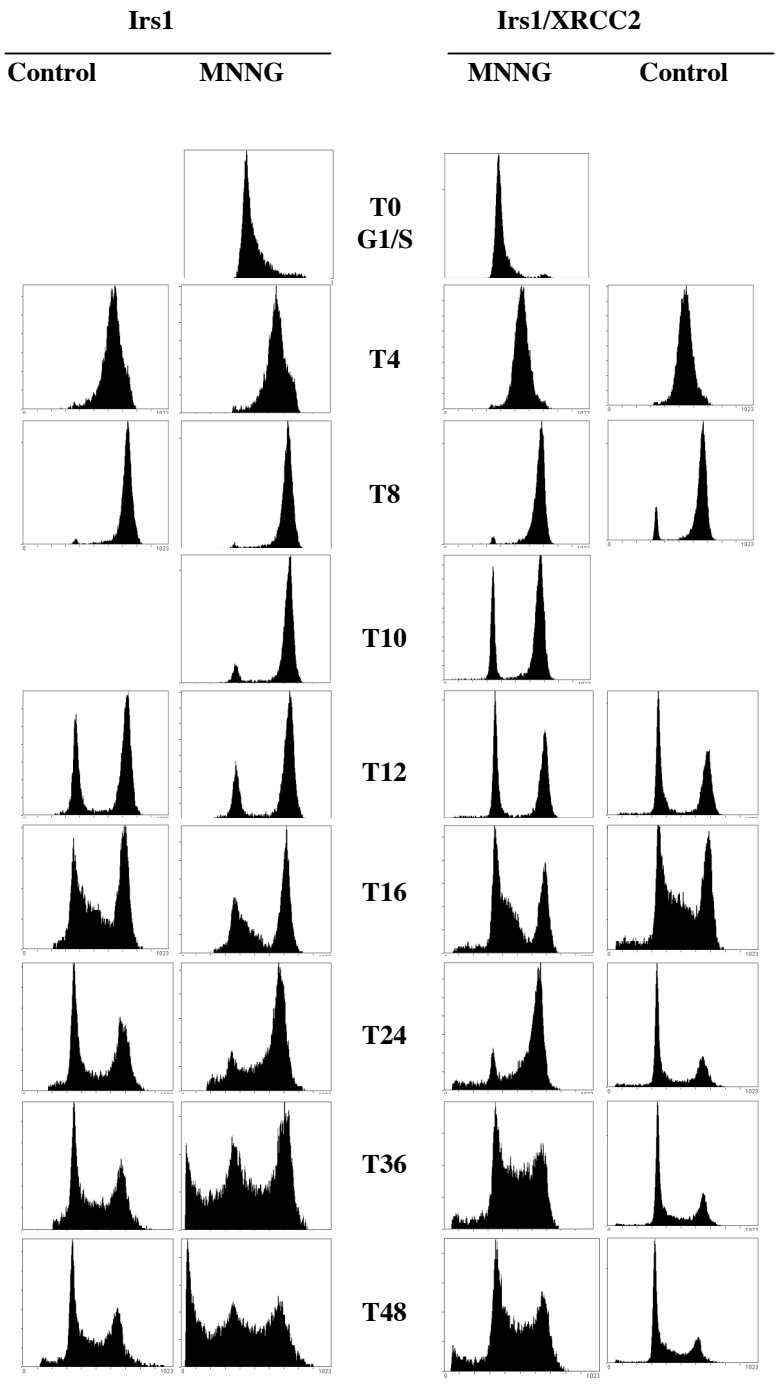
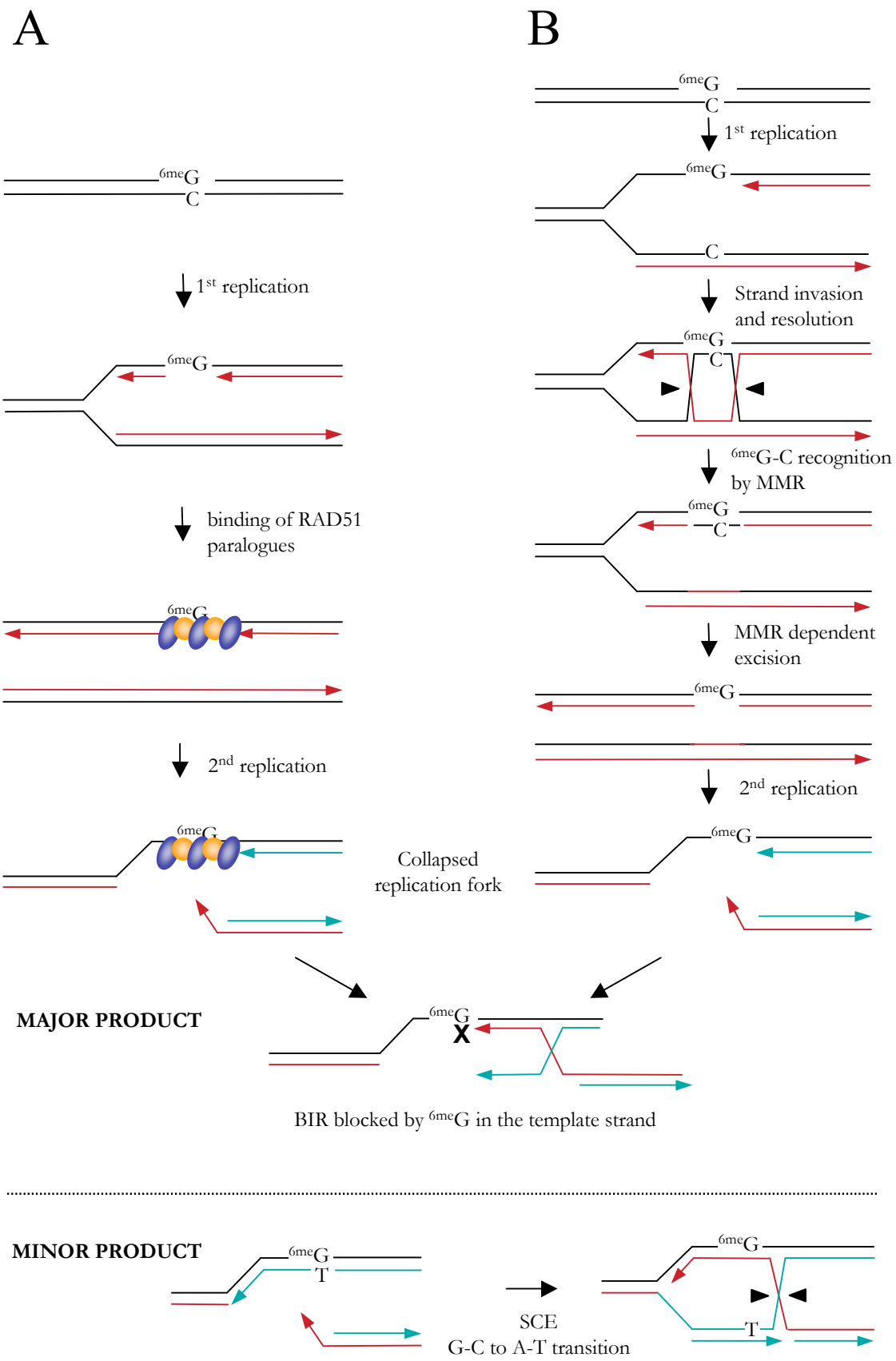

Irs1
Irs1/XRCC2
Control
MNNG
MNNG
Control
T0
G1/S
T4
T8
T10
T12
T16
T24
T36
T48

Figure 5


A
B
6meG
C
1st replication
Strand invasion
and resolution
6meG-C recognition
by MMR
binding of RAD51
paralogues
MMR dependent
excision
2nd replication
Collapsed
replication fork
MAJOR PRODUCT
X
BIR blocked by 6meG in the template strand
MINOR PRODUCT
T
SCE
G-C to A-T transition

4.4 Homologous recombination rescues mismatch-repair-dependent cytotoxicity of S_N1-type methylating agents in S. cerevisiae

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Current Biology

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MMR is highly conserved, from bacteria to humans and the sensitivity to ^{6me}G –inducing agents is one of its hallmarks. The finding that the MMR status did not affect the sensitivity of *Saccharomyces cerevisiae* to MNNG was unexpected. One possible explanation for this difference was differential ability of yeast cells to process the secondary lesions created by MMR during attempted processing of ^{6me}G residues. As was shown for mammalian cells, homologous recombination contributes to cell survival after MNNG treatment. Taking into account that recombination efficiency in yeast cells is much higher than in mammalian cells, this could be a pathway responsible for MNNG resistance in yeast. Indeed, HR inactivation sensitized *S. cerevisiae* to MNNG and, as in human cells, defects in the MMR genes MLH1 and MSH2 rescued this sensitivity. This finding suggests the importance of secondary pathways involved in MMR-mediated cytotoxicity.

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Homologous Recombination Rescues Mismatch-Repair-Dependent Cytotoxicity of S_N1-Type Methylating Agents in *S. cerevisiae*

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Summary

Resistance of mammalian cells to S_N1-type methylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) generally arises through increased expression of methylguanine methyltransferase (MGMT), which reverts the cytotoxic O^6-methylguanine (^{Me}G) to guanine, or through inactivation of the mismatch repair (MMR) system, which triggers cell death through aberrant processing of ^{Me}G/T mispairs generated during DNA replication when MGMT capacity is exceeded [1]. Given that MMR and ^{Me}G-detoxifying proteins are functionally conserved through evolution, and that MMR-deficient *Escherichia coli dam*$^-$ strains are also resistant to MNNG [2], the finding that MMR status did not affect the sensitivity of *Saccharomyces cerevisiae* to MNNG [3] was unexpected. Because ^{Me}G residues in DNA trigger homologous recombination (HR) [4–7], we wondered whether the efficient HR in *S. cerevisiae* might alleviate the cytotoxic effects of ^{Me}G processing. We now show that HR inactivation sensitizes *S. cerevisiae* to MNNG and that, as in human cells, defects in the *MMR* genes *MLH1* and *MSH2* rescue this sensitivity. Inactivation of the *EXO1* gene, which encodes the only exonuclease implicated in MMR to date [8, 9], failed to rescue the hypersensitivity, which implies that scExo1 is not involved in the processing of ^{Me}G residues by the *S. cerevisiae* MMR system.

Results and Discussion

In the yeast *Saccharomyces cerevisiae*, the functional homolog of human MGMT, scMgt1p, has been shown to remove methyl groups from ^{Me}G residues and, to a lesser extent, from O^4-methylthymine [10], and to protect the cells from both mutagenesis and killing induced by MNNG [3]. The key players in postreplicative MMR are the MutS homologs scMsh2p, scMsh3p, and scMsh6p and the MutL homologs scMlh1p and scPms1p (functional homolog of the human PMS2). These polypeptides are also functionally highly related to the human proteins, yet the MMR status of *S. cerevisiae* was reported not to influence the response of *mgt1* mutants to methylating agents [3]. This difference between the lower and higher eukaryotic cells is unlikely to be due to the lack of apoptosis in yeast because mammalian cells can be efficiently killed by MNNG without having to activate the machinery of programmed cell death [11] and because even *dam*$^-$ MMR-deficient *E. coli* are more resistant to killing by this methylating agent than MMR-proficient strains [2]. We therefore reasoned that the different response of yeast and mammalian cells to methylating agents might be explained either by differences in DNA-damage recognition or else by differences in other pathways of methylation-damage processing.

The human mismatch binding factor hMSH2/hMSH6 (hMutSα) has been reported to bind oligonucleotide substrates containing ^{Me}G/T or ^{Me}G/C mispairs [12], and we wanted to test whether the yeast proteins behaved similarly. To this end, we overexpressed the *S. cerevisiae* MMR-recognition factor scMsh2p/scMsh6p (scMutSα) and purified it to near homogeneity (Figure 1A). Gel-shift assays with increasing amounts of scMutSα (1.7–67 nM) and a constant amount (6.6 nM) of ^{Me}G/T-, ^{Me}G/C-, G/T- and G/C-containing oligonucleotide duplexes (Figures 1B–1E) revealed that all DNA substrates formed slowly migrating protein/DNA complexes at high (>30 nM) scMutSα concentrations. Formation of these nonspecific (ns) complexes has been observed previously [13] and probably results from aggregation of scMsh2p/scMsh6p heterodimers on the same oligonucleotide substrate. In contrast, the ^{Me}G/T and G/T substrates formed a more rapidly migrating specific (s) complex with the heterodimer; this complex already appeared at low protein concentrations and represented ~90% of the total labeled oligonucleotide duplex at 16.4 nM protein concentration (lane with an asterisk in Figures 1B and 1C). In case of the ^{Me}G/C substrate, only a small amount of the specific complex was formed at lower protein concentrations (~35% at 16.4 nM scMutSα, Figure 2E, lane with an asterisk), but this amount was higher than that formed by the G/C homoduplex substrate (~10%, see Figure 2D, lane with an asterisk). In both latter cases, the nonspecific band was also apparent at the 16.4 nM scMutSα concentration, indicating a weak recognition of the ^{Me}G/C substrate. These results were confirmed in competition assays, in which the labeled G/T heteroduplex (6.6 nM) in the presence of scMutSα (16.4 nM) was competed with a 10-fold excess of the unlabeled ^{Me}G/T, ^{Me}G/C, G/T, or G/C duplexes. In these experiments, the ^{Me}G/T oligonucleotide appeared to be an even better substrate for scMutSα than G/T (Figure 1F), although it should be pointed out that the affinity of the mismatch binding heterodimer for the methylated oligonucleotides is highly dependent on sequence context, which is generally not the case for G/T (our unpublished observations). Differences in DNA-damage recognition thus cannot explain the difference in phenotype between yeast and mammalian cells. We therefore argued that these differences must lie in the processing of methylation damage downstream from damage recognition.

Treatment of mammalian and yeast cells with MNNG was reported to give rise to elevated levels of homologous recombination (HR). Furthermore, in human cells,

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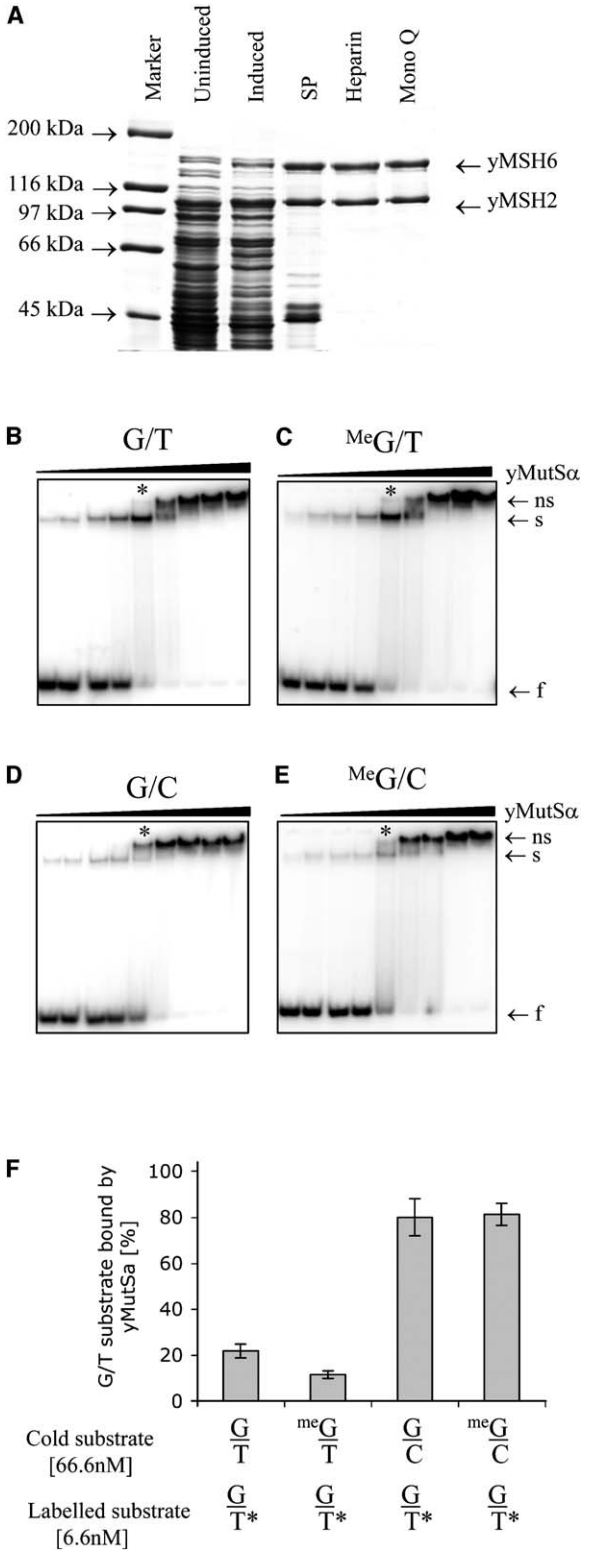

Figure 1. Substrate Specificity of the *S. cerevisiae* Mismatch Binding Factor Msh2p/Msh6p

(A) Overexpression of scMsh2p/Msh6p in *E. coli*. Uninduced, total extract from BL21 codon plus cells; Induced, total extract from BL21 codon plus cells overexpressing scMsh2p/Msh6p; SP, Heparin and Mono Q, pooled fractions eluted from the respective FPLC chromatography columns. The figure shows aliquots from different purification steps separated on a denaturing 7.5% polyacrylamide gel stained with Coomassie Blue.

(B–E) Binding of the scMsh2p/Msh6p heterodimer to different DNA substrates. Formation of specific (s) and nonspecific (ns) complexes. The heterodimer (1.7–67 nM) was incubated with ^{32}P-labeled 6.6 nM G/T, ^{Me}G/T, G/C, or ^{Me}G/C DNA substrates. The lane with 16.4 nM protein concentration is indicated by an asterisk. The protein/DNA complexes were analyzed by a gel-shift assay as described in the Experimental Procedures and visualized by autoradiography. (E) Competition binding assay. The Msh6p/Msh2p heterodimer (16.4 nM) was incubated with 6.6 nM ^{32}P-labeled G/T heteroduplex. The preformed complexes were then challenged with a 10-fold excess of unlabeled G/T, ^{Me}G/T, G/C, and ^{Me}G/C DNA substrates. The fraction of the labeled G/T substrate bound by Msh2p/Msh6p was quantitated by Typhoon 9600 PhosphoImager with ImageQuant software.

HR was elevated specifically in an MMR-dependent manner [4–7]. Given that the efficiency of HR in *S. cerevisiae* is substantially higher than in mammalian cells, we wondered whether this could be the underlying cause of the different responses of these organisms to methylating agents. Inactivation of the *RAD52* gene, which is required for most HR processes in *S. cerevisiae*, rendered the cells hypersensitive to MNNG. Additional inactivation of the *MMR* genes *MSH2* or *MLH1* had no effect on sensitivity, which was not particularly surprising given that these cells expressed scMgt1p (Figure 2A). However, when the *MGT1* gene was also inactivated, the *mgt1 rad52* double mutant became exquisitely sensitive to MNNG, whereas the *mgt1 rad52 msh2* and *mgt1 rad52 mlh1* triple mutants were sensitized to a substantially lesser extent (Figure 2B). It therefore appears that MMR-mediated processing of ^{Me}G residues gives rise to cytotoxic intermediates that are resolved by homologous recombination. Due to the high efficiency of HR in yeast, these intermediates are most likely successfully repaired, which would account for the substantial resistance of MMR-proficient yeast cells to MNNG. Interestingly, the survival curve of the *mgt1 rad52* strain appears to be biphasic (Figure 2B). At low MNNG concentrations (0.5–1.5 μM), the inactivation of *MSH2* or *MLH1* fully suppresses the sensitivity of the *mgt1 rad52* strain to MNNG, which shows that the killing is at this concentration range linked almost exclusively to the processing of ^{Me}G residues by the MMR system. In contrast, cell death at high MNNG concentrations (>3 μM) is also most likely caused by other types of damage, such as strand breaks arising through processing of *N*-methylated purines and abasic sites, which account for more than 90% of the damage caused by these agents. Moreover, overexpression of scMgt1p in the *rad52* strain failed to improve survival at high MNNG concentrations (data not shown), which further supports the hypothesis that the cytotoxicity is in this case linked to DNA modifications distinct from ^{Me}G. A similar situation was also observed in human cells [14].

It is well established that both scMsh2p and scMlh1p are absolutely required for MMR, whereas the mechanism and players in the downstream events of the repair process remain enigmatic. We thus decided to examine the involvement of the *EXO1* gene, which en-

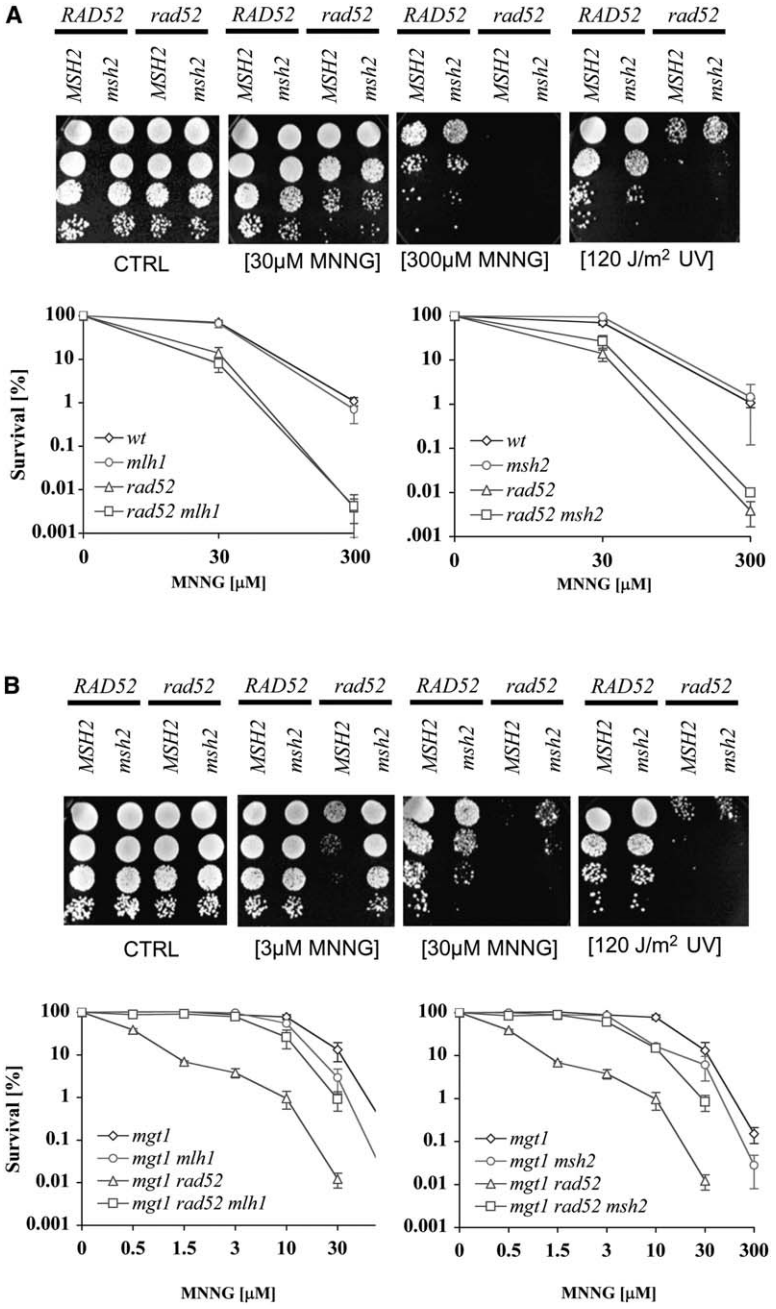

Figure 2. MNNG-Induced Killing of *S. cerevisiae* Strains with Different Genetic Backgrounds

(A) Comparison of MNNG sensitivities of the MMR-deficient (*mlh1* or *msh2*) and/or recombination-deficient (*rad52*) strains as measured by the spot test. Mid-log phase cells were treated with the indicated concentrations of MNNG, harvested and spotted on YPD plates at proper serial dilutions as described in the Experimental Procedures. (Wild-type genes are labeled with capitals, deletion mutants with lowercase letters) Sensitivity to ultraviolet (UV) light was used as a control. The upper panel shows a representative experiment, and the lower panel shows a graphic representation of data pooled from 3–5 independent experiments. Error bars show standard error of the mean.

(B) Effect of methylguanine methyltransferase (*mgt1*) deficiency on the sensitivity of MMR- and/or recombination-deficient strains to MNNG. Sensitivity to UV light was used as a control. The upper panel shows a representative experiment, and the lower panel shows a graphic representation of data pooled from 3–5 independent experiments. Error bars show standard error of the mean.

codes the only exonuclease implicated in MMR to date (with the notable exception of the proofreading activity of polymerase delta [8, 15]) in the processing of methylation damage induced by MNNG. In contrast to *MSH2* and *MLH1* inactivation, which rescued the hypersensitive phenotype of the *mgt1 rad52* mutant strain, deletion of the *EXO1* gene brought about a further, albeit minor, increase in MNNG sensitivity (Figure 3). These results suggest that scExo1p helps the cell overcome the deleterious effects of DNA methylation, rather than being involved in the MMR-dependent cytotoxic processing of ^{Me}G residues. The role of the scExo1p in MMR has been the subject of some discussion. The protein plays a role in several other biological processes, including mutation-avoidance pathways distinct from MMR, telomere integrity, and processing of double-strand breaks prior to homologous recombination, and it is likely that its functions overlap with those of other exonucleases [16]. Although our data provide evidence that scExo1p is not required for the processing of methylation damage, they fail to indicate which exonuclease (if any) fulfills this role in vivo.

The interaction between yeast MMR and HR in the processing of DNA damage has been described previously [17]. In that study, disruption of *MMR* genes conferred a mild but significant (1.5- to 6-fold) resistance to cisplatin, carboplatin, and doxorubicin, and contrary to our results, the resistant phenotype was de-

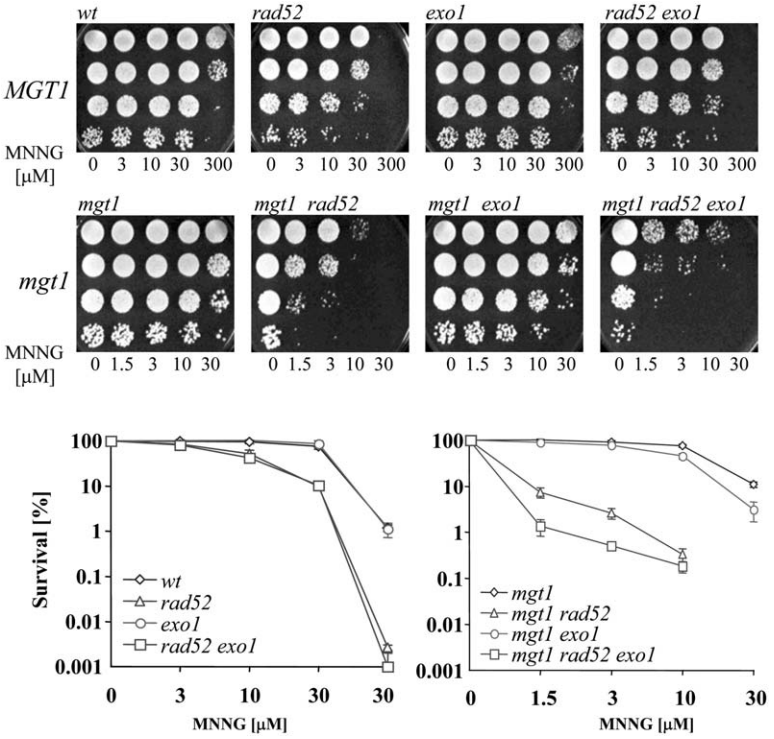

Figure 3. MNNG Sensitivities of Recombination- (*rad52*), Methylguanine Methyltransferase- (*mgt1*), and/or Exonuclease 1 (*exo1*)-Deficient *S. cerevisiae*

Mid-log phase cells of the indicated strains were treated with the indicated concentrations of MNNG and spotted at proper serial dilutions on YPD plates as described in the Experimental Procedures. The upper panel shows a representative experiment, and the lower panel shows a graphic representation of data pooled from 3–5 independent experiments. Error bars show standard error of the mean.

pendent on functional scRad52p. This difference can be explained. DNA lesions induced by the above agents block DNA replication and require recombination for efficient lesion bypass [17]. Because MMR controls the fidelity of recombination processes, it most likely lowers recombination frequency in DNA containing these bulky adducts and thus augments cytotoxicity. In contrast, ^{Me}G/C pairs arising in methylguanine-methyltransferase-deficient cells upon methylation do not block DNA synthesis per se and are well tolerated in the absence of MMR. Our model for the cytotoxicity of S_N1-type methylating agents is outlined in Figure 4. In the absence of MMR, DNA replication will give rise to one unmodified progeny DNA molecule and one that carries the ^{Me}G residue paired either with thymine of cytosine, neither of which needs to be resolved by recombination. In the presence of MMR, the ^{Me}G/C or ^{Me}G/T mispairs arising during replication are detected by the scMsh2/scMsh6 heterodimer, which triggers a round of repair. However, because MMR is directed to the newly synthesized DNA strand, the modified nucleotide persists in the template strand. Because the polymerase filling the repair patch cannot find a perfect base-pairing partner for ^{Me}G, it may leave a gap or a similar lesion that could be repaired by HR. In the absence of HR, the gap would be converted during the next replication round to a double-strand break that might trigger cell-cycle arrest and cell death.

In summary, we show that damage reversal by scMgt1p, along with methylation damage processing and repair by HR, mask the sensitivity of MMR-proficient *S. cerevisiae* cells toward killing by S_N1-type methylating agents. Inactivation of MMR in the *mgt1 rad52* yeast background rendered cells approximately 20-fold more tolerant to killing by MNNG Recombination-deficient *S. cerevisiae* thus resemble the methylation-sensitive phenotype of mammalian cells [18]. The lack of involvement of scExo1p in the processing of methylation damage implies that the exonuclease function is fulfilled by another enzyme(s). Given the amenability of *S. cerevisiae* to genetic manipulation and high-throughput screening, our present results should help us design assays for identification of these enzymes, as well as of other gene products involved in the processing of methylation damage. It is hoped that at least some of these findings will help us understand the mode of action of S_N1 methylating agents, which represent an important class of cancer chemotherapeutics.

Experimental Procedures

Production of scMsh2-scMsh6 in *E. coli*

The pET11a-scMSH2-scMSH6 plasmid (a kind gift of M. Hingorani) was transformed into BL21 DE3 codon plus cells (Strategene). The induction and purification was performed essentially as described [19], except that ultrasonic treatment was used for cell disruption.

Oligonucleotide Substrates

The 34-mers 34TopG (AATTCCCGGGGATCCGTCGACCTGCAGCC AAGCT), 34Top^{Me}G (AATTCCCGGGGATCCGTC^{Me}GACCTGCAGC CAAGCT), 34BottomT (AGCTTGGCTGCAGGTTGACGGATCCCCG GGAATT) and 34BottomC (AGCTTGGCTGCAGGTCGACGGATCC CCGGGAATT) were synthesized by Microsynth (Balgach, Switzerland) and purified by polyacrylamide gel electrophoresis (the sites of mismatch or base modification are underlined). Despite the fact that Microsynth strictly adhered to the protocol for ^{Me}G incorporation into the oligonucleotide, as recommended by the manufacturer of the corresponding phosphoramidite (Glenn Research, USA), mass-spectrometric analysis by NanoESI revealed that a substantial part of the ^{Me}G-containing oligonucleotide still contained the

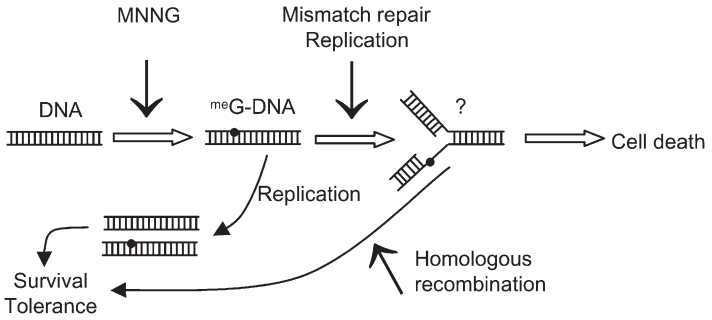

Figure 4. Model of MMR-Induced Killing upon Treatment with Methylating Agents

See text for details.

isobutyryl protecting group on the exocyclic nitrogen of the methylated guanine. The fully deprotected oligonucleotide had to be separated from the contaminant by reverse-phase HPLC, for which a Nucleosil 100Å, C_{18}, 5 μm, 250 × 4.0 mm column (BGB Analytik) eluted with a linear gradient of 5%–20% acetonitrile in 100 mM triethylamonium acetate (pH 7) was used. The "bottom" oligonucleotides were radioactively labeled with [γ-^{32}P]-ATP (Amersham Biosciences) and polynucleotide kinase (New England Biolabs) and purified on Sephadex G25 columns. Three pmol of the labeled "bottom" oligonucleotides were then annealed with 4.5 pmol of the cold top oligonucleotides in 1× polynucleotide kinase buffer by brief heating to 95°C and slow cooling to room temperature. In this way we obtained the duplex substrates G/C, G/T, ^{Me}G/C, and ^{Me}G /T.

Gel-Shift Experiments

The indicated amounts of purified *S. cerevisiae* Msh2p/Msh6p protein (diluted where necessary in 50 mM NaCl, 25 mM Tris.HCl [pH 8.0]) and 6.6 nM of the labeled substrate in binding buffer (10% glycerol, 0.33 mg/ml BSA, 25 mM HEPES [pH 8.0], 0.5 mM EDTA, and 0.5 mM DTT) were incubated for 20 min at room temperature in a total volume of 30 μl. The reaction products were separated on 6% polyacrylamide gels (acrylamide:bisacrylamide ratio 19:1) at room temperature at 150V for 85 min. The gels were dried and scanned with the Typhoon 9400 imager and ImageQuant TL software (both Amersham Biosciences).

Yeast Media

For unselective growth, YPD medium (2% glucose, 2% bactopeptone, and 1% yeast extract) was used. Clones where the gene of interest was replaced by the KANMX cassette were selected on YPD plates supplemented with 200 μg/ml G418 (Invitrogen). Where necessary, the media were solidified by 2% agar (Difco). All yeast strains were propagated under aerobic conditions at 30°C.

Yeast Strains and Transformation

The yeast strains used in this study were all isogenic derivatives of FF18733 and FF18734 *S. cerevisiae* strains (a kind gift of F. Fabre)

Table 1. *S. cerevisiae* Strains Used in This Study

Strain	Relevant Genotype	Source
FF18733	MATa; leu2-3, 112; ura3-52; his7-2; lys1-1, trp1-289	F. Fabre
ZS30	FF18733 with *msh2::KANMX*	Z. Storchova
EP82	FF18733 with *mlh1::KANMX*	E. Papouli
FPC 45	FF18733 with *rad52::URA3*	this study
FPC 1-1	FF18733 with *mgt1::KANMX*	this study
FPC 50	FF18733 with *exo1::KANMX*	this study
FPC 32	FF18733 with *mgt1::KANMX; rad52::URA3*	this study
FPC 24	FF18733 with *rad52::URA3; msh2::KANMX*	this study
FPC 37	FF18733 with *rad52::URA3; mlh1::KANMX*	this study
FPC 3-3b	FF18733 with *mgt1::KANMX; msh2::KANMX*	this study
FPC15	FF18733 with *mgt1::KANMX; mlh1::KANMX*	this study
FPC 52	FF18733 with *rad52::URA3; exo1::KANMX*	this study
FPC 55	FF18733 with *mgt1::KANMX; exo1::KANMX*	this study
FPC 21	FF18733 with *mgt1::KANMX; rad52::URA3; msh2::KANMX*	this study
FPC 61	FF18733 with *mgt1::KANMX; rad52::URA3; exo1::KANMX*	this study
FPC 39	FF18733 with *mgt1::KANMX; rad52::URA3; mlh1::KANMX*	this study
FF18734	MATα; leu2-3, 112; ura3-52; his7-2; lys1-1, trp1-289	F. Fabre
ZS30-1d	FF18734 with *msh2::KANMX*	this study
EP 85	FF18734 with *mlh1::KANMX*	E. Papouli
FF18743	FF18734 with *rad52::URA3*	F. Fabre
FPC 2-1	FF18734 with *mgt1::KANMX*	this study
FPC 51	FF18734 with *exo1::KANMX*	this study
FPC 30	FF18734 with *mgt1::KANMX; rad52::URA3*	this study
FPC 28	FF18734 with *rad52::URA3; msh2::KANMX*	this study
EP 95	FF18734 with *rad52::URA3; mlh1::KANMX*	E. Papouli
FPC 3-2a	FF18734 with *mgt1::KANMX; msh2::KANMX*	this study
FPC 16	FF18734 with *mgt1::KANMX; mlh1::KANMX*	this study
FPC 54	FF18734 with *rad52::URA3; exo1::KANMX*	this study
FPC 58	FF18734 with *mgt1::KANMX; exo1::KANMX*	this study
FPC 22	FF18734 with *mgt1::KANMX; rad52::URA3; msh2::KANMX*	this study
FPC 59	FF18734 with *mgt1::KANMX; rad52::URA3; exo1::KANMX*	this study
FPC 43	FF18734 with *mgt1::KANMX; rad52::URA3; mlh1::KANMX*	this study

and are listed in Table 1. Replacement of the *MMR* genes was performed with *kanMX4* replacement cassettes with specifically designed primers (see below), with pUG6 (*MSH2*, *MGT1*, and *EXO1*) or pFA6a-*kanMX4* (*MLH1*) plasmids being used as templates for polymerase chain reactions (PCR), essentially as described [20]. The primer sequences for gene disruption were as follows (forward, reverse primer):

MSH2, GACACTCTACTCCAATATCAACTGTAAAAAATCTCTTTATCTGCTGGACCTAACATCAAAATCCTCAGATTAAAAGGAGCTGAAGCTTCGTACGC, CTTTCCAATGCATATTATATGTACTATTTGTATCTATATATTATCTATCGATTCTCACTTAAGATGTCGTTGTAATATTAATTATAACAACGCATAGGCCACTAGTGGATCTG;

MLH1, ATAGTGATAGTAAATGGAAGGTAAAAATAACATAGACCTATCAATAAGCACAGCTGAAGCTTCGTACGC, AAAGGAAAGGGCATACACTTTCAAATGAAACACAATCACACTCAGGAAATGCATAGGCCACTAGTGGATCTG;

MGT1, TGGCAGGGCATTTAAAATGCGGTGGAAACAAGGAAGATTAATCAAGTAATGATATAGCATCAGCTGAAGCTTCGTACGCTGCAG, CAATTTACCACATATACATAACTATTTCTTATGTTTATTTTCCTAAAATCCTTTATCCAAGCATAGGCCACTAGTGGATCTG;

EXO1, TGCTTTTTGGACCACATTAAAATAAAAGGAGCTCGAAAAAACTGAAAGGCGTAGAAAGGACAGCTGAAGCTTCGTACGCTGCAG,TTCGACGAGATTTTCATTTGAAAAATATACCTCCGATATGAAACGTGCAGTACTTAACTTGCATAGGCCACTAGTGGATCTG.

The transformations were performed by the lithium-acetate method. The genotypes of all strains used were verified by PCR (primer sequences and details are available on request), Southern blotting, and tetrad analysis.

Spot Tests

A stock solution of 1 M MNNG (Sigma) was prepared in DMSO and stored in the dark at –20°C. Because of the estimated ~45 min half-life of MNNG in aqueous solutions, all experiments were performed in liquid cultures as follows: The cells were inoculated from a YPD plate into 3 ml of liquid YPD medium and cultivated overnight. The cells were then diluted 1:15 with YPD, and 3 ml cultures were incubated for a further 3 hr, when the cells were again in an exponential growth phase. The cells were then mock-treated and treated with several concentrations of freshly diluted MNNG for 45 min. They were harvested, washed, and spotted (~12 μl drops) at serial dilutions on YPD plates. For UV treatments, the cells from the untreated cultures were spotted at similar dilutions on several YPD plates and subsequently irradiated with the indicated doses (UV Stratalinker 1800). The plates were evaluated after 3 days of cultivation at 30°C. Because no significant differences were observed between the a and α mating types, the results shown are based on 3–5 independent experiments carried out with strains in both a and α backgrounds.

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5 CONCLUSIONS AND PERSPECTIVES

Mismatch repair is highly conserved from bacteria to man. It increases the fidelity of DNA replication by ~1000 fold through correcting the errors that escaped the proofreading activity of the replicative polymerases. The importance of functional MMR has been highlighted by the discovery that defects in the correction of replication errors are linked to hereditary non-polyposes colorectal cancer (HNPCC), as well as significant proportion of sporadic colon cancers. Besides maintaining genome stability by correcting replication errors, MMR also participates in other DNA transactions such as homologous recombination. Of particular interest to clinical applications is the finding that MMR-deficient cells display resistance to certain types of DNA damaging agents used in chemotherapy. The most prominent effect has been observed with S_N1-type methylating agents, where MMR-deficient cells can tolerate ~100-fold higher concentrations than MMR-proficient ones.

So far, studies of MMR-dependent DNA metabolism relied on cell lines, which were either derived from MMR-deficient tumours or were selected by long-term treatments that favoured loss of MMR activity. Such cell lines have high mutation rates and long term passaging thus causes a genetic drift from their MMR-proficient counterparts. Establishment of a cell line in which expression of a single MMR protein, namely MLH1, can be regulated by doxycylin is an important contribution to the field. Cejka et al. characterized 293T Lα cells and showed that only a full complementation of MLH1 brings about G_2/M arrest upon MNNG treatment, although MMR proficiency was achieved with suboptimal levels of the protein. This finding has important implications when considering treatments of MMR-proficient tumours. Absence of cell cycle arrest and associated cell death favours tumour progression and accumulation of additional mutations.

Cytotoxicity of S_N1-type methylating agents has been attributed to formation of 6-methyguanine (^{6me}G) residues in the DNA. Under normal conditions, the methyl group can be rapidly removed by detoxifying enzyme MGMT. If left unreapired, it is addressed by the MMR machinery during subsequent replication. The mechanism that transforms ^{6me}G into a cytotoxic lesion has been the subject of many studies, but the precise role of MMR has been controversial. One hypothesis suggested that MMR proteins have a direct signalling role, and that formation of ^{6me}G -containing mispairs can be sensed by the

MutSα-MutLα complex, thus transmitting the signal downstream to the checkpoint machinery. The nature of the cell cycle arrest after MNNG treatment is contradictory to this hypothesis and Stojic *et al.* showed that additional processing of the initial lesions is required in order to activate cell cycle checkpoint responsible for the sustained arrest.

In vivo activation of ATM was MMR-dependent but not required for the arrest, indicating that another kinase, ATR, could play a role in the activation of the checkpoint. Indeed, the ATR-CHK1 pathway was shown to be required for the MMR-dependent arrest. The late onset of the cell cycle arrest after MNNG treatment indicates that 6-methylguanine *per se* is not sufficient for the checkpoint activation; rather, it requires two cell cycles to exert its cytotoxic effect. The lesions created by MMR and the subsequent processing were shown to contain ssDNA as indicated by formation of RPA and RAD51 foci, as well as sustained persistence of single strand breaks in comet assays.

Next, we showed that the recognition of MNNG–induced damage by MutSα occurs in the first S phase, during which decoupling of replication and repair foci becomes apparent. Although MMR recognizes ^{6me}G-containing mispairs already in the first cell cycle, persistence of ^{6me}G in the DNA is required throughout the two cell cycles to activate the checkpoint. We showed that in order for the cells to pass to the second cell cycle, homologous recombination is required for the processing of the lesions generated during the first S phase. Absence of efficient homologous recombination arrests cells at the first G_2/M boundary and has a dramatic influence on overall survival. The finding that the cytotoxicity of a single lesion can be attributed to more than one repair mechanism highlights the importance of multi-pathway activation. Processing of ^{6me}G adducts by MMR creates substrates for homologous recombination, which in turn fails to repair the intermediates, thus causing cell death. From the clinical perspective, this finding can be of interest, because inactivation of homologous recombination could enhance the cytotoxic effect of methylating agents, thus allowing the use of lower doses, with less severe side-effects.

The use of biophysical methods will be of extreme importance in the charcaterisation of structures of DNA intermediates that cause cytotoxicity.

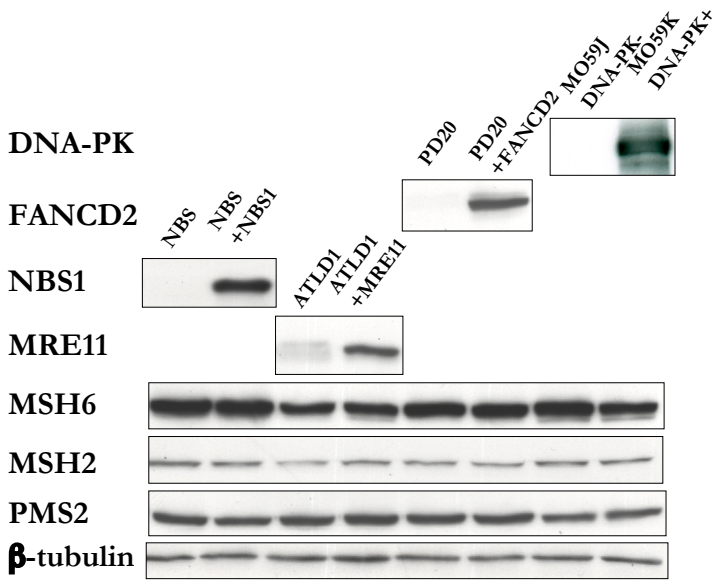
The finding that homologous recombination rescues DNA intermediates created by MMR-dependent processing after MNNG treatment was shown to be even more pronounced in yeast. Recombination-proficient yeast cells are highly resistant to MNNG treatment, even so that the difference between MMR-proficient and deficient cells cannot be discovered. This paradigm was explained when recombination-deficient yeast

cells were shown to be highly sensitive to killing by MNNG, and that inactivation of MMR rescued this sensitivity.

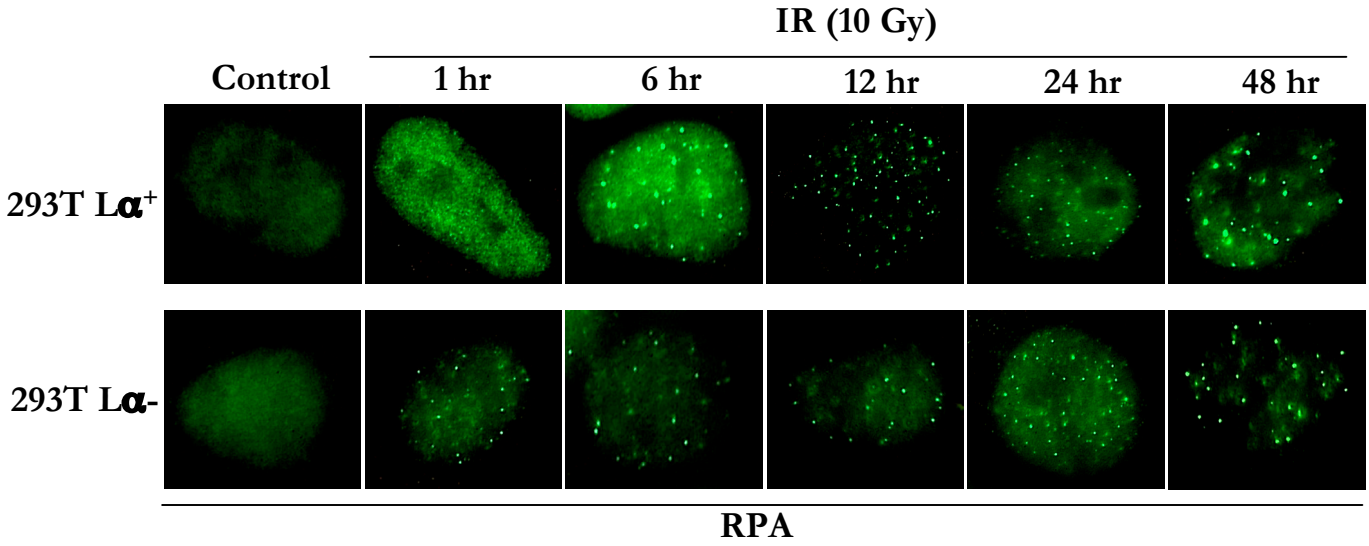
The establishment of a yeast system provides invaluable tool for further genetic tests, with the goal of identifying novel components of both the MMR pathway and the recombination machinery. The existence of an analogy with the human mechanism offers a solid base for these future studies.

6 APPENDIX

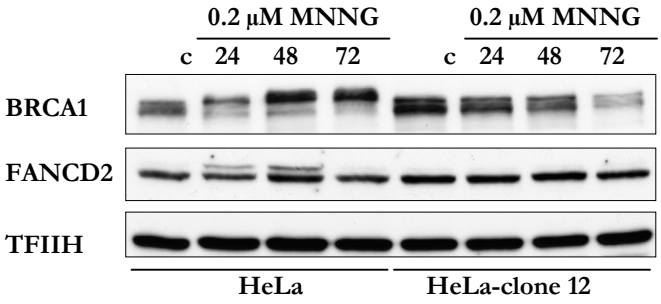
Unpublished results



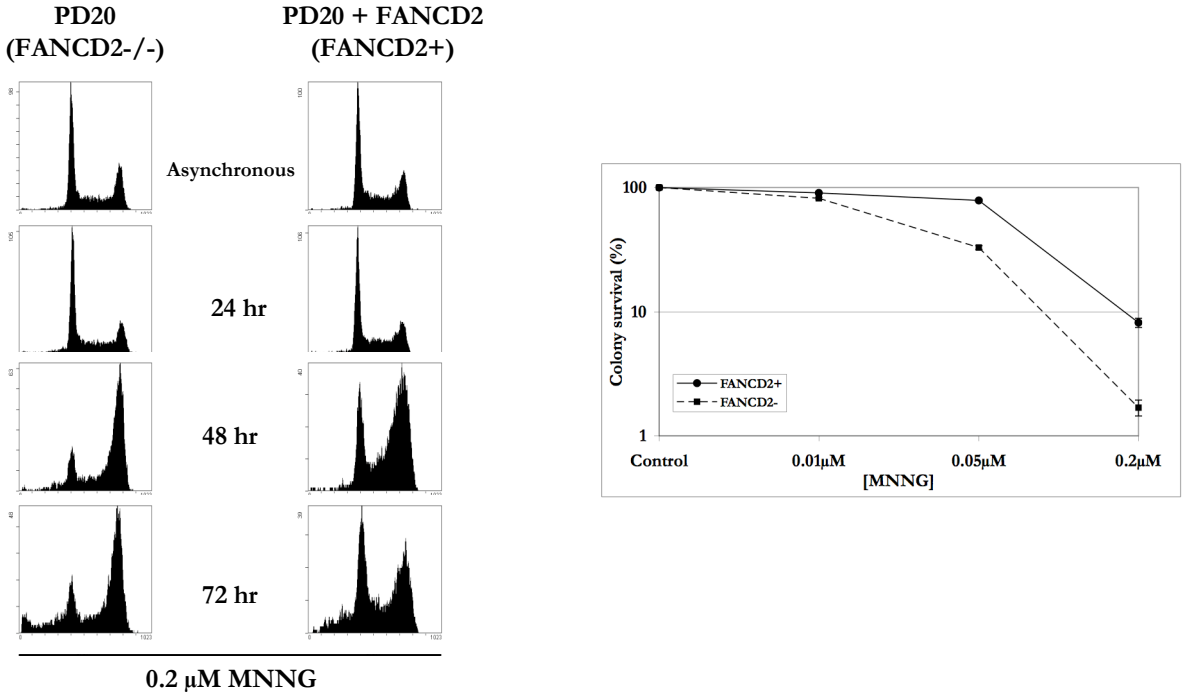
MMR status of the cell lines used during the study. Western-blot for MMR proteins in DNA-PK cells (MO59J and MO59K), FANCD2 cells (PD20 and PD20+FANCD2), NBS cells (NBS and NBS+NBS1) and ATLD1 cells (ATLD1 and ATLD1+MRE11). All the cell lines were shown to express MMR proteins and were MMR-proficient.



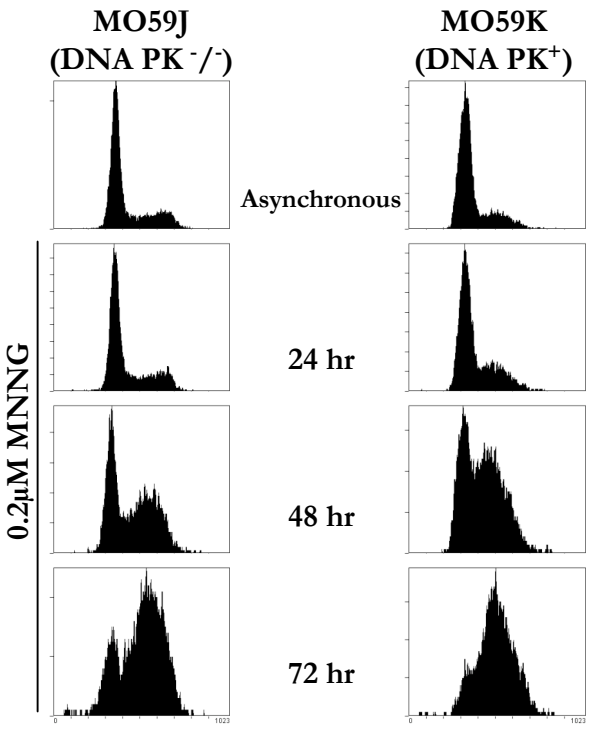
Kinetics of the formation of RPA foci in 293T Lα cells upon ionazing radiation (IR).
Unlike upon MNNG treatment, where relocalisation of RPA into foci was MMR dependent, IR-induced foci formed independently of MMR status.



MMR-dependent activation of BRCA1 and FANCD2 upon MNNG treatment. HeLa-clone 12, due to the loss of PMS2, is a MMR-deficient cell line. It was selected from parental HeLa cells through long-term treatment with MNNG.
Activation of BRCA1 was observed only in MMR-proficient cells, already 24 hours after treatment. The slower migrating form represents phosphorylated BRCA1 protein. BRCA1 is involved in homologous recombination through its interaction with RAD51, as well as signalling downstream to the checkpoint machinery.
Monoubiquitylation of FANCD2 is a consequence of activation of Fanconi anaemia pathway and occurs in MMR-proficient cells upon MNNG treatment. The monoubiquitylated form of FANCD2 is actively recruited to the chromatin, where it is thought to be engaged in repair processes, particularly homologous recombination.



The Fanconi anaemia pathway is involved in MMR-dependent response upon MNNG treatment.
Cell cycle analysis and survival of FANCD2-proficient and -deficient cell lines upon MNNG treatment. Checkpoint activation and subsequent arrest is more pronounced in FANCD2-deficient cell line, indicating its requirement for the processing of DNA intermediates. This requirement is highlighted in cell survival, pointing out that the Fanconi anaemia pathway is involved in the repair of cytotoxic lesions created by MMR-dependent processing.



Cell cycle distribution of DNA-PK cells upon MNNG treatment. As a major player in NHEJ pathway, DNA-PK might be involved in the repair of possible DSB intermediates created by MMR processing of ^{6me}G residues. Cell cycle arrest was shown to be identical in these two non-isogenic cell lines, indicating the absence of, or a minor role of DNA-PK in the cell survival upon MNNG treatment.

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- 7th Charles Rodolphe Brupbacher Symposium, Advances in Oncology: Advances in Oncology: From Model Systems to the Clinic, March 16-19, 2005, Zurich, **Switzerland**

PUBLICATIONS:

Mojas N, Stojic L, Jiricny J. Recombinational processing of MMR induced lesions after MNNG treatment, Manuscript submitted.

Cejka P, Mojas N, Gillet L, Schaer P, Jiricny J. Homologous Recombination Rescues Mismatch Repair-Dependent Cytotoxicity of SN1-type Methylating Agents in S. cerevisiae**. Curr Biol. 2005 Aug 9;15(15):1395-400**

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